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Epigenetic dysregulations of miR-30a and LINE-1 in gastric cancer

위암에서 miR-30a와 LINE-1의 후성적 조절에 관한 연구

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민지민

Abstract

Epigenetic dysregulations of miR-30a and LINE-1 in gastric cancer

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Cancer Biology

Background: Epigenetic alterations such as microRNA (miRNA) and DNA methylation can regulate cancer cell properties in gastric cancer. miRNAs are an abundant class of negative gene regulators that control a wide range of biological functions such as cellular proliferation, differentiation and apoptosis by regulating multiple gene targets. miR-30a has been reported as a downregulated miRNA in gastric cancer, but its biological function and clinical implication have not been reported much. DNA methylation can repress transposable elements such as long interspersed nuclear element-1 (LINE-1), and LINE-1 is frequently hypomethylated in cancer. LINE-1 retrotransposition can generate many small RNAs such as miRNAs, and LINE-1 silencing is associated with miR-30a.

Purpose: The aim of this study was to identify miR-30a expression and LINE-1 methylation patterns as well as miR-30a biological function by finding its gastric

cancer-specific target genes. We also checked whether both miR-30a and LINE-1 could be diagnostic or prognostic markers in gastric cancer by assessing the clinical impacts.

Method: We performed qRT-PCR using our tissue samples to identify miR-30a and its target gene expressions. We used data from TCGA and NCBI GEO, to confirm their expressions in open source databases. To determine their biological functions, miR-30a was overexpressed by mimics or inhibited by inhibitors in gastric cancer cell lines. Moreover, a stable cell line overexpressing miR-30a was used for the *in vivo* tumorigenesis assay. Microarray was introduced to confirm the gastric cancer-specific target gene of miR-30a, which was later knocked down by siRNA for functional studies. For LINE-1 methylation analysis, we examined four CpG sites of LINE-1 by quantitative bisulfite pyrosequencing using frozen and formalin-fixed paraffin-embedded (FFPE) tissues of gastric cancer. Finally, we analyzed the clinicopathological data.

Result: In gastric cancer tissues, miR-30a was down-regulated, and LINE-1 was hypomethylated when compared to normal tissues. Ectopic expression of miR-30a decreased cell growth, migration capacity and colony formation *in vitro* and *in vivo*. Furthermore, we found that miR-30a directly targeted the ITGA2 gene and that the miR-30a-ITGA2 axis was significantly related to *H. pylori*-infected and microsatellite instability (MSI)-high gastric cancer. Higher expression of ITGA2 was particularly exhibited in intestinal type gastric cancer than in diffuse type gastric cancer. When we used both frozen and FFPE tissues for LINE-1 methylation analysis, LINE-1 was differentially methylated between two types of tissues. In frozen tissues, LINE-1 methylation status was different according to gender, differentiation,

lymphatic and venous invasions. In FFPE tissues, LINE-1 methylation was significantly different according to tumor location and venous invasion.

Conclusion: Cumulatively, miR-30a functions as a tumor suppressor by directly targeting ITGA2. miR-30a-ITGA2 axis is related to several clinicopathological features of gastric cancer such as *H. pylori*, MSI and intestinal type. LINE-1, as one of miR-30a regulators, can be a marker according to its methylation status for several clinicopathological parameters, and especially it can be a marker for venous invasion in irrespective of gastric cancer tissue types. Therefore, we suggest that both miR-30a-ITGA2 axis and LINE-1 may be useful strategies for treatment and prediction of prognosis in gastric cancer. LINE-1 study was published in *Molecules and Cells* in 2017, and figures and tables were reproduced and used in this paper [1].

Key word: Epigenetic alteration, miR-30a, tumor suppressor, ITGA2, LINE-1, venous invasion, gastric cancer

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Introduction

1. Gastric cancer and Epigenetic dysregulation

Gastric cancer is the fifth most-common cancer and third leading cause of cancer-related mortality worldwide [2]. Especially in Asian countries such as Korea, Japan and China, it is the second most common cause of cancer-related death [3]. Etiologically, gastric carcinogenesis can be induced by virus infection such as Helicobacter pylori (*H. pylori*) and Epstein-Barr, or hereditary and environmental factors are involved in the cause of gastric cancer [4]. Gastric cancer has been classified into several subtypes based on molecular characterization to encompass gastric cancer heterogeneity for clinical use [5, 6]. Also, to predict gastric cancer risk and prognosis, gene- or protein-based biomarker studies have been actively doing [7-9]. However, it is limited to develop comprehensive biomarker because the molecular mechanisms are complex and still poorly understood.

Epigenetic alterations in cancer are mainly as follows; (i) Histone modifications such as methylation, acetylation and phosphorylation, (ii) DNA methylation, (iii) microRNA (miRNA) and non-coding RNA (ncRNA) [10]. Among these factors epigenetically affecting cancer, miRNAs, which are small non-coding RNA molecules consist of about 22 nucleotides, have been known as one of the most important biomarker related to gastric cancer progression and prognosis [11]. miRNAs function as regulators of transcriptional and translational silencing, then they repress gene expression [12, 13]. In cancer, miRNA dysregulation can be occurred during each step of its biogenesis by genetic and epigenetic alterations, hypoxia, mutations and so on [14]. Dysregulated miRNAs function as tumor suppressors or oncogenes in pre- or post-transcriptional silencing by binding 3’ UTR

region of its target mRNAs. Aberrant biogenesis of tumor suppressive miRNAs regulates cancer cell properties such as proliferation, angiogenesis and invasion by targeting oncogenic mRNAs [15]. For example, miR-200, miR-34 and let-7 are characterized as well-known tumor suppressors targeting cell fate-related genes or metastasis-inducing genes [16].

Retrotransposons can transcribe DNA to RNA via reverse transcription, then reintegrate identical DNA sequences into the genome. They are class I transposable elements which consist of long terminal repeats (LTRs) and non-LTRs including long interspersed nuclear element-1 (LINE-1), and non-LTRs are the majority of human transposable elements and affect human genome [17]. Many small RNAs including miRNAs are derived from repetitive sequences and retrotransposition activity of LINE-1, and these retrotransposon-derived small RNAs regulate gene expressions in specific tissues [18, 19]. Retrotransposition of LINE-1 can generate a significant number of small RNAs in the human genome [20]. LINE-1 constitutes a substantial portion (~17%) of the human genome [21]. LINE-1, which has two open reading frames, ORF1p and ORF2p, functions as a regulator of somatic retrotransposition, transcriptome effects and DNA damage [22]. Among epigenetic factors affecting cancer, DNA methylation in promoter CpG islands is associated with several processes such as repression of transposable elements and contribution to carcinogenesis by transcriptional silencing. So far, LINE-1 methylation has been reported to be associated with various features of cancer.

2. miR-30a and LINE-1 in cancer

In the miRNA microarray data from four studies, miR-30a-5p has been one of downregulated miRNAs in gastric cancer [11, 23-25]. This miRNA functions as a suppressor of tumor growth via direct inhibition of *DTL*, *MTDH* or *SEPT7* in colon cancer, breast cancer or glioma [26-28], and it also suppresses metastasis via targeting *PIK3CD, ERG, MTDH* or *vimentin* in colorectal, prostate or breast cancers [28-31]. In gastric cancer, a particular miRNAs signature including miR-30a-5p was useful to predict gastric cancer patient survival, with low risk score of miR-30a-5p [32]. In precancerous stage, it was reported that miR-30a was downregulated in intestinal metaplasia, and it caused overexpression of metaplastic marker genes through *HNF4γ* regulation [33]. In cancerous stage, only one target gene of miR-30a, vimentin, which is respective of metastasis was identified [34].

miR-30 family consists of five members, miR-30a, -30b, -30c, -30d and -30e, and they have identical seed sequences. All other members except miR-30a have been also reported as negative regulators of tumor growth and metastasis in various type of cancers such as breast cancer, colorectal cancer, ovarian cancer and prostate cancer [28, 35-38]. In gastric cancer, miR-30b and miR-30c modulate cell migration and invasion by targeting *EIF5A2* and *MTA1* [39, 40]. Therefore, we focused on miR-30a-5p as one of critical tumor suppressors in gastric cancer.

LINE-1 silencing increases several miRNAs including let-7 family, miR-196a, miR-30a/d, miR-191 and miR-200c in human breast cancer cells [41]. In addition, both LINE-1 and miR-30a are useful prognostic markers for gastric cancer [42]. Therefore, we additionally studied LINE-1 methylation as one of miR-30a regulatory factors in gastric cancer.

LINE-1 is often hypomethylated in cancers such as colon, bladder, breast and gastric cancer, and its hypomethylation is related to tumor progression and poor prognosis [43-46]. More concretely, in colorectal cancer, global LINE-1 hypomethylation is inversely correlated with microsatellite instability (MSI) and worse overall survival [47-49], and it also triggers liver metastasis by inducing the *MET* proto-oncogene [50].

In gastric neoplasia categorized according Vienna classification, LINE-1 was differentially methylated between low-grade dysplasia, high-grade dysplasia and intramucosal cancer, and it especially hypomethylated in high-grade dysplasia and intramucosal cancer [51]. LINE-1 hypomethylation has been reported to be associated with prognostic indicators in gastric carcinogenesis [44], and its methylation level can be changed on progressing from intestinal metaplasia to gastric adenoma, affecting gastric cancer poor prognosis [52].

In a meta-analysis study including gastric cancer, LINE-1 is significantly hypomethylated in cancer patients compared to controls when it is analyzed using fresh/frozen and formalin-fixed paraffin-embedded (FFPE) tissue samples, but its methylation level is not different using blood samples [53]. Previously, LINE-1 methylation analysis using 38 frozen and 434 FFPE tissue samples showed that LINE-1 methylation status significantly different between fresh/frozen tissues and FFPE tissues, and LINE-1 methylation in FFPE tissues was significantly associated with several clinicopathological features such as intestinal type, lymphatic invasion and venous invasion [54].

However, thus far, it has not been studied to investigate the relation between clinicopathological features and LINE-1 methylation status in frozen tissue samples

of gastric cancer. Therefore, we analyzed LINE-1 methylation using both FFPE and frozen tissues to evaluate whether LINE-1 methylation can be an epigenetic marker for several clinicopathological parameters such as differentiation, tumor location, lymphatic invasion, venous invasion and lymph node metastasis.

Part I.

Downregulated miR-30a promotes cancer cell properties by targeting integrin alpha2 (ITGA2) in gastric cancer

Purpose of this study

We hypothesized that miR-30a-5p can function as a tumor suppressor by regulating cancer cell properties through targeting several oncogenes in gastric cancer. Therefore, the aim of this study is to reveal miR-30a-5p expression pattern and biological function in gastric cancer and to find the miR-30a-5p target genes based on microarray technique. Also, the present study is to assess clinical impacts of miR-30a-5p-target gene axis by discovering their functions in gastric cancer. Through these investigations, we would like to check whether the miR-30a-5p can use a diagnostic or prognostic marker in gastric cancer.

Material and method

Cell lines and tissue specimens

Four human gastric cancer cell lines, AGS, SNU-216, SNU-601 and MKN28, and human embryonic kidney (HEK293) cells were obtained from the Korean Cell Line Bank (Seoul, Korea) and maintained in RPMI 1640 (Welgene, Daegu, Korea) with 10% Fetal bovine serum (Gibco, Invitrogen, UK) and 1% penicillin-streptomycin (Gibco, Invitrogen, UK). HSC44Luc, HSC45-M2 and 44As3Luc cell lines were provided by K Yanagihara, National Cancer Center Research Institute, Japan. They were cultured and maintained in the conditions same as above gastric cancer cell lines. Thirty pairs of primary gastric cancer tissues and matched normal mucosa were used to analyze miRNA or mRNA expression levels. All tissue specimens were obtained from Seoul National University Hospital, Korea. The clinicopathological features of the patients are summarized in Table 1. The present study was approved by the Institutional Review Boards (IRB) of Seoul National University Hospital (IRB No. 1308-122-517).

Table 1. Clinicopathological features of 30 patients with gastric cancer

Characteristic	Gastric cancer (n=30)	Percentage
Gender		
Male	22	73.33%
Female	8	26.67%
Age, years		
≤ 61	14	46.67%
> 61	16	53.33%
T classification		
T1	2	6.67%
T2	4	13.33%
T3	11	36.67%
T4	13	43.33%
N classification		
N0	8	26.67%
N1	3	10.00%
N2	9	30.00%
N3	10	33.33%
Distance metastasis		
Absent	23	76.67%
Present	7	23.33%
TNM stage		
I	6	20.00%
II	8	26.67%
III	7	23.33%
IV	9	30.00%
Lauren Classification		
Intestinal	18	60.00%
Diffuse	10	33.33%
Mixed	2	6.67%
WHO classification		
Differentiated	16	53.33%
Undifferentiated	14	46.67%

Tumor location		
Upper	8	26.67%
Middle	10	33.33%
Lower	12	40.00%
Lymphatic invasion		
Not identified	10	33.33%
Present	18	60.00%
Unknown	2	6.67%
Venous invasion		
Not identified	23	76.67%
Present	5	16.67%
Unknown	2	6.67%
Perineural invasion		
Not identified	14	46.67%
Present	14	46.67%
Unknown	2	6.67%

Computational analysis

We used public databases such as the NCBI Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA). miRNA and mRNA expression data were obtained by RNA sequencing (RNAseq) of each database. In the GEO database, we used miRNA expression data of 8 non-cancer and 60 primary gastric cancer (GSE26595). In TCGA, miRNA expression data of 42 normal and 476 stomach cancer was generated using the Illumina GA HiSeq, and gene expression data of 37 normal and 384 stomach cancer was generated using the Illumina HiSeq. Also, prediction programs such as TargetScan, PITA and miRanda were applied for identifying candidate target genes of miR-30a.

Transfection using miR-30a mimic, miR-30a inhibitor, stable miR-30a-expressing vector and ITGA2 siRNA

For transient induction or inhibition of miR-30a, we transfected miR-30a mimics or miR-30a inhibitor with their negative controls (Bioneer, Korea) into gastric cancer cells. To establish gastric cancer cell line stably expressing miR-30a, the full-length coding region of miR-30a cDNA was amplified from normal genomic DNA and cloned into the pmR-ZsGreen1 vector (Clontech, Japan). Stable cell line was generated by transfection of plasmids into SNU-601 cells and selection of miR-30a-expressing clones by Geneticin (G418). Primers are described in Table 2. For transient knockdown of ITGA2, siRNAs for ITGA2 (Bioneer, Korea) were transfected into gastric cancer cell lines. Transient transfections of miRNA or si-RNA were performed using Lipofectamine RNAiMAX (Invitrogen), and plasmid

transfection was carried out using Lipofectamine 3000 (Invitrogen) following the manufacturer's instructions.

Cell proliferation, soft agar colony formation and migration assays

Cell proliferation assay was performed in AGS, SNU-601, SNU-216 and SNU-668 cell lines using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) at absorbance 450nm for 72 h. For soft agar colony formation assay, base agar were seeded into 6-well plates, and transfected cells (1×10^4 cells/well) with top agar were seeded on the base agar at triplicate. The plates were incubated for 4 weeks. Colonies were stained with Crystal violet (Sigma-Aldrich, St. Louis, Missouri, USA) and counted under the microscope.

Migration assay was performed with Boyden chambers (BD Biosciences) that had 8um pore size membranes. Cells were seeded into upper chamber (2×10^5 cells/well). After 18 h, cells were stained with Crystal violet (Sigma-Aldrich, St. Louis, Missouri, USA) and counted under the microscope to calculate relative migration rates.

Microarray

SNU-601, HSC44Luc and 44As3Luc cells were transfected with miR-30a mimic or NC mimic using RNAiMAX reagent (Invitrogen). Total RNA was extracted by TRIzol reagent (Invitrogen). RNA purity and integrity were evaluated by ND-1000 Spectrophotometer (NanoDrop, Wilmington, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). Total RNA was amplified and purified

using TargetAmp-Nano Labeling Kit for Illumina Expression BeadChip (EPICENTRE, Madison, USA) to yield biotinylated cRNA according to the manufacturer's instructions. Briefly, 500 ng of total RNA was reverse-transcribed to cDNA using a T7 oligo(dT) primer. Second-strand cDNA was synthesized, *in vitro* transcribed and labeled with biotin-NTP. After purification, the cRNA was quantified using the ND-1000 Spectrophotometer (NanoDrop, Wilmington, USA). 750 ng of labeled cRNA samples were hybridized to each Human HT-12 v4.0 Expression Beadchip for 17h at 58°C, according to the manufacturer's instructions (Illumina, Inc., San Diego, USA). Detection of array signal was carried out using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK) following the bead array manual. Arrays were scanned with an Illumina bead array Reader confocal scanner according to the manufacturer's instructions. The quality of hybridization and overall chip performance were monitored by visual inspection of both internal quality control checks and the raw scanned data. Raw data were extracted using the software provided by the manufacturer (Illumina GenomeStudio v2011.1 (Gene Expression Module v1.9.0)). Array probes transformed by logarithm and normalized by quantile method.

Luciferase reporter assay

A fragment containing ITGA2 3'UTR was amplified and cloned into the pmirGLO Dual-Luciferase miRNA target expression vector (Promega, USA). Primers are described in Table 2. Luciferase reporter vectors were transfected into the cells, and luciferase activity was measured using the Dual-Luciferase® Reporter Assay System following the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cell lines and tissue samples using Trizol reagent (Invitrogen, Carlsbad, USA) or miRNeasy Mini Kit (QIAGEN). cDNA was synthesized to analyze both mRNA and microRNA expressions using a TOP script cDNA synthesis kit (Enzynomics, Daejeon, Korea) or PrimeScript RT reagent kit (Takara) according to the manufacturer's protocols. The expression level of miR-30a was measured using SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA) by normalizing to the levels of U6. Also, the mRNA expression levels were quantified by normalizing to GAPDH. Primers are indicated in Table 2. All the reaction were performed and analyzed by comparative △△Ct methods using Step One Plus Real-Time PCR system (Applied Biosystems, Carlsbad, CA).

Western blotting

Cells were washed with cold PBS and lysed with RIPA buffer (ThermoFisher Scientific, MA, USA) plus protease inhibitor cocktails (Roche). Proteins were isolated from cell lysates, and western blotting was performed using anti-ITGA2 (1:2000, Abcam, Cambridge, UK), anti-α-tubulin (1:1000, Sigma-Aldrich, St. Louis, Missouri, USA).

Table 2. Primers for stable miR-30a expressing vectors, gene expression analysis by real-time PCR and luciferase reporter assay

Gene		Sequence
Hsa-miR-30a cloning	Forward	GTGGCTAACAATAATGAATGAAACC
	Reverse	TGAAGCCCTCTAAAAATGTACAGAC
Hsa-miR-30a-5p	RT	CTCAACTGGTGTCGTGGAGTCGGCA ATTCAGTTGAGCTTCCAGT
	Forward	ACACTCCAGCTGGGTGTAAACATCC TCGA
	Reverse	TGGTGTCGTGGAGTCG
Human ITGA2	Forward	CCTACAATGTTGGTCTCCCAGA
	Reverse	AGTAACCAGTTGCCTTTTGGATT
Human FBXO45	Forward	AGTTGGTGTTCTCTTACCTGGA
	Reverse	CCTCGCTGTTCTCATCGCC
Human ADAM19	Forward	GAGGGGCGAGAACTGATCCT
	Reverse	TGGTTTGAGGGTTACCACTTGA
Human SEC23A	Forward	GGAGTCCGATTTAGTTGGAATGT
	Reverse	AGGTCTCTCTTTCAGTGGTGT
ITGA2 3’ UTR	Forward	ATCTTTAAACTGGCTGGCCCAGAGT TTACATTCT
	Reverse	TCGAAGAATGTAAACTCTGGGCCAG CCAGTTTAAAGATAGCT
ITGA2 Mutated 3’ UTR	Forward	ATCTTTAAACTGGCTGGCCCAGAGA AAAAATTCT
	Reverse	TCGAAGAATTTTTTCTCTGGGCCAG CCAGTTTAAAGATAGCT

In vivo experiments

Female BALB/c nu/nu mice, at age of 6-8 weeks (Orient Bio., Sungnam, Korea), were used to establish tumor xenograft models. For tumorigenicity assay, we subcutaneously injected empty vector transfected (n=8) or stably miR-30a overexpressed (n=8) SNU-601 cells into the right flank of nude mice. 1×10^7cells in 200 μl of medium were injected into each mice. Tumor size was measured using calipers twice every week, and the tumor volume was calculated by lenghth (L) × width (W) × height (H). At day 63, all mice was sacrificed, and tumor mass was pathologically checked by hematoxylin and eosin (H&E) staining. This animal experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of Clinical Research Institute at Seoul National University Hospital (14-0183-C1A0(1)).

Tissue microarray

For evaluation of the expression profiles of ITGA2 protein, a collection of 450 gastric adenocarcinomas resected at SNUH in 2004 (SNUH-2004-GC; SuperBioChips) was used. Immunohistochemical staining was performed automatically using OPTIVIEW universal DAB kit on Ventana BenchMark XT Staining systems (Ventana Medical Systems). Tumor tissues were selected in each core, and a scoring was performed based on a staining intensity. The staining was considered as positive if more than 10% of cellular staining was observed in each core. The staining intensity was as follows: 0, negative; 0.5, faint positive; 1, weak positive; 2, moderate positive; 3, strong positive.

Statistical analysis

Student t-test was used to compare gene expression and functions such as cell proliferation and migration. *In vivo* experiment, two-way ANOVA was tested to evaluate tumor volume changes. To assess correlation between miR-30a and target genes, the Spearman's correlation test was conducted. These statistical analyses were performed using GraphPad Prism V5.0 (GraphPad Software, San Diego, CA). A *p*-value was considered as statistically significant when it was less then 0.05. Significant differences were indicated by asterisks: $*p < 0.05$, $** p < 0.01$, $*** p < 0.001$.

For microarray, statistical significance of the expression data was determined using Independent T-test and fold change in which the null hypothesis was that no difference exists among groups. False discovery rate (FDR) was controlled by adjusting p value using Benjamini-Hochberg algorithm. For a differentially expressed gene (DEG) set, Hierarchical cluster analysis was performed using complete linkage and Euclidean distance as a measure of similarity. Gene-Enrichment and Functional Annotation analysis for significant probe list was performed using DAVID (http://david.abcc.ncifcrf.gov/home.jsp). All data analysis and visualization of differentially expressed genes was conducted using R 3.0.2 (www.r-project.org).

Result

Expression level of miR-30a-5p in gastric cancer

We analyzed expression patterns of miRNA family in gastric cancer using RNA sequencing data of stomach adenocarcinoma from The Cancer Genome Atlas (TCGA). Among them, expressions of several miR-30 family members were distinct between normal and tumor. Unlike other members, miR-30a and miR-30e were particulary highly expressed in normal, and their low expressions in lots of tumor were shown (Figure 1 and Table 3). We performed quantitative real-time PCR using our gastric cancer tissue cohort. miR-30a expression was validated in 30 pairs of gastric cancer tissues, and it was significantly down-regulated in 25 pairs of gastric cancer tissues compared to matched normal mucosa (Figure 2A). We also applied another computational analysis using public data, NCBI Gene Expression Omnibus (GEO) database, with TCGA data. TCGA data showed that miR-30a is quite down-regulated in gastric tumor (n=476) compared with normal (n=42), and NCBI GEO data indicated the same pattern (gastric adenocarcinoma; n=60, non-cancer; n=8) (Figure 2B and 2C). Therefore, we suggest that miR-30a-5p is a potential tumor suppressor gene in gastric cancer.

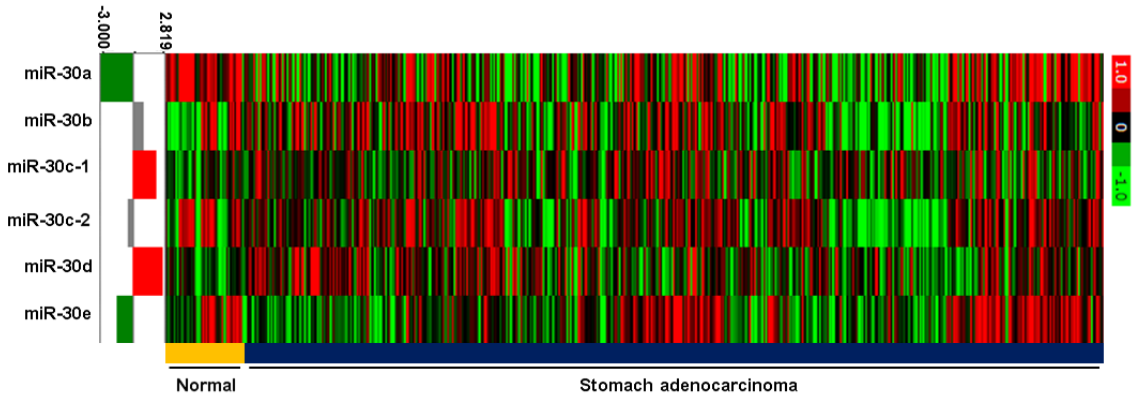


Figure 1. Expression of miR-30 family in normal and stomach adenocarcinoma from RNA sequencing data of TCGA

Table 3. Expressions of miR-30 family in TCGA

miR-30 family	Normal Tissue (n=42)	Primary Tumor (n=476)	*P* value
miR-30a	14.36 ± 0.10	13.52 ± 0.05	< 0.001
miR-30b	8.30 ± 0.17	8.53 ± 0.04	0.106
miR-30c-1	1.01 ± 0.07	1.27 ± 0.03	0.008
miR-30c-2	8.93 ± 0.13	8.81 ± 0.03	0.291
miR-30d	11.96 ± 0.07	12.29 ± 0.03	0.002
miR-30e	13.24 ± 0.10	13.01 ± 0.03	0.031

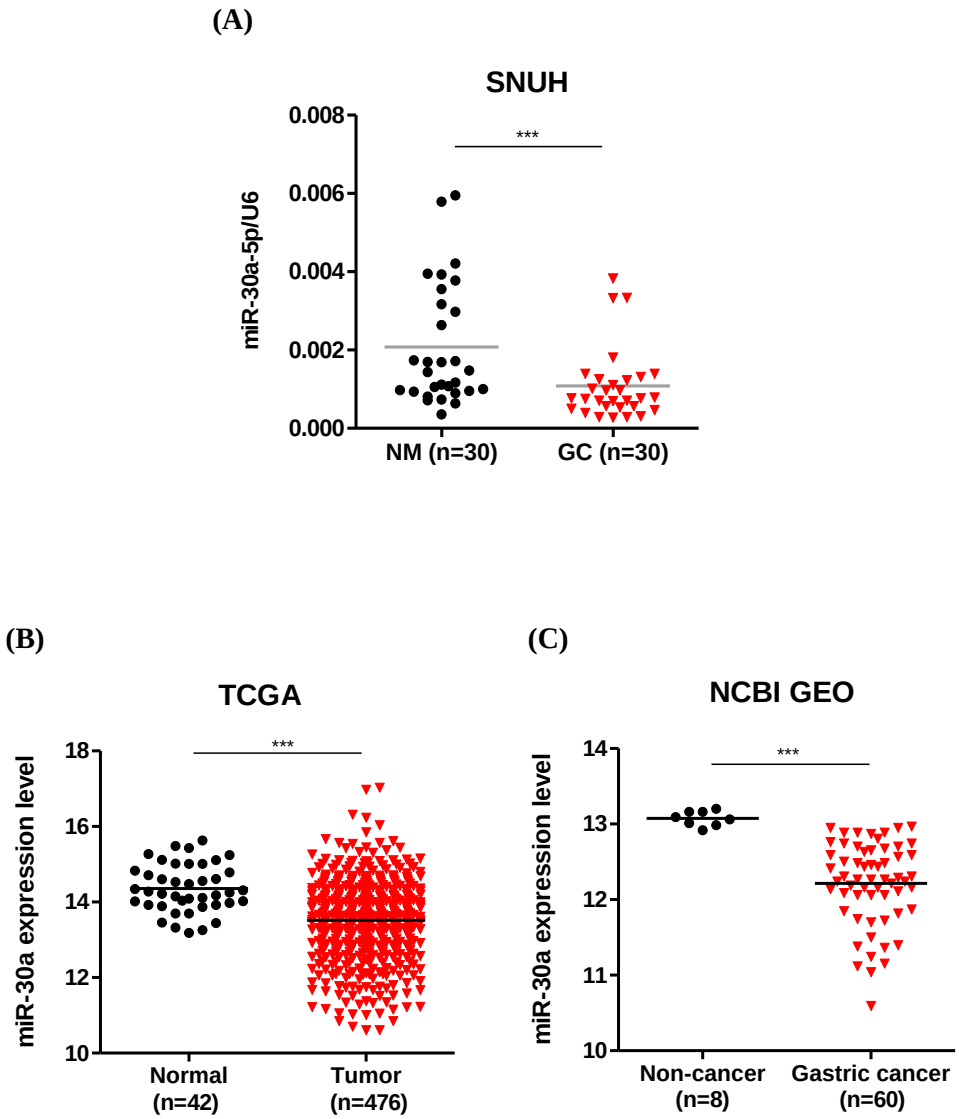


Figure 2. miR-30a expression between normal and tumor tissues in our cohort (A), TCGA (B) and NCBI GEO (C)

miR-30a can function as a tumor suppressive miRNA in gastric cancer

In gastric cancer cell lines, it was expressed differentially between 6 cell lines (Figure 3A), so we treated miR-30a mimic or inhibitor in these cells for further *in vitro* study to test whether miR-30a can function as a tumor suppressor gene in gastric cancer. As a control, negative controls of miR-30a mimic or miR-30a inhibitor were treated in the same conditions. The induction or inhibition of miR-30a was confirmed as shown in Figure 3B and 3C.

Cell proliferation was reduced when we treated miR-30a mimic (Figure 4). Soft agar colony formation assay showed similar result, and the differences were statistically significant. In contrast, cells treated with miR-30a inhibitor formed much more colonies than its control group (Figure 5). Based on these data, we subcutaneously injected SNU-601 cells stably overexpressing miR-30a or empty vector into BALB/c nude mice. Each group consisted of eight mice. During 63 days, tumor volume had been significantly reduced in miR-30a group than in control group before sacrificed (Figure 6). Thus, it shows that miR-30a effectively suppressed tumorigenesis of gastric cancer cells both *in vitro* and *in vivo*.

To confirm the effects of miR-30a on cell movements, cells treated with negative control or miR-30a mimic were observed in time-based monitoring after cell seeding in Boyden chambers. The number of migrated cells through Boyden chamber was significantly decreased in miR-30a mimic treated group compared to negative control (Figure 7). These results implies that this miRNA is an effective factor to inhibit cell migration in gastric cancer.

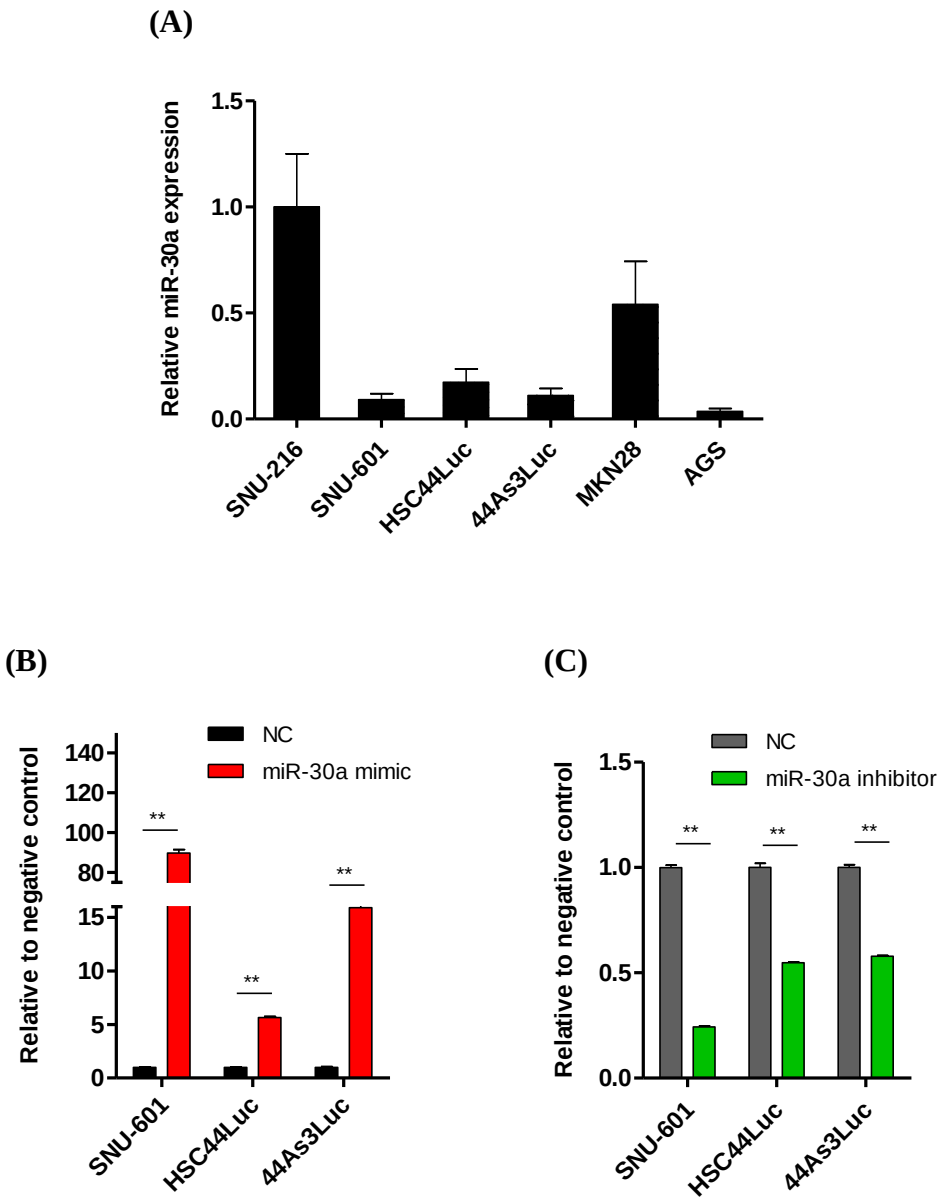


Figure 3. Relative expression level of miR-30a-5p in gastric cancer cell lines. Expression screening using 6 gastric cancer cell lines (A) and its relative expression level after miR-30a mimic or miR-30a inhibitor treatments compared negative control (NC) (B)

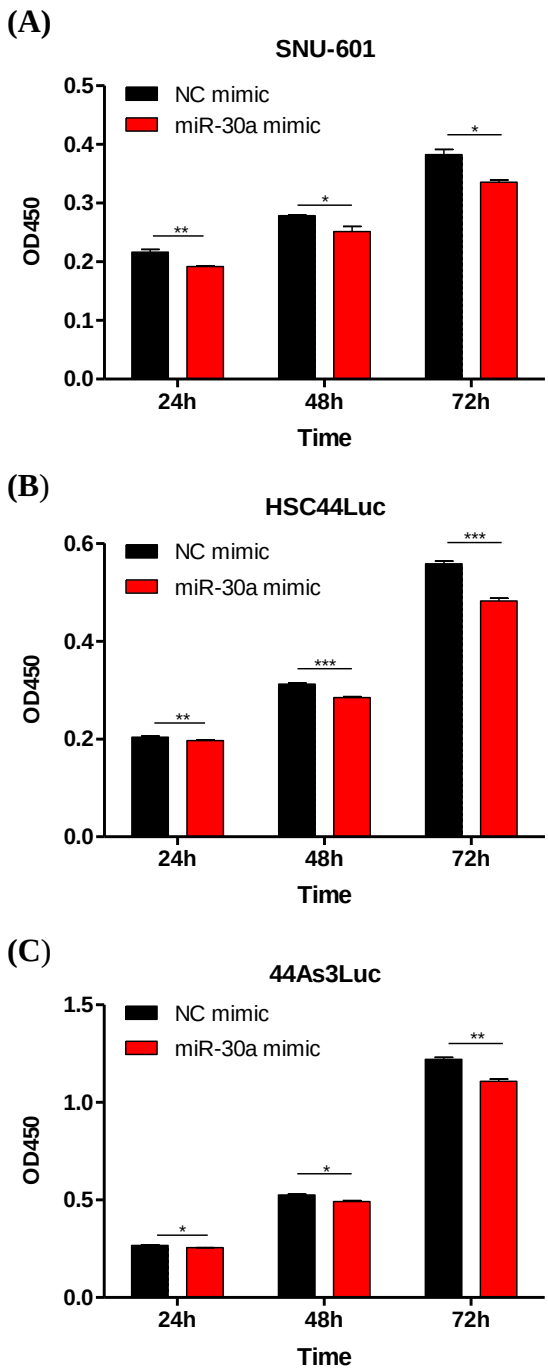


Figure 4. Cell proliferation assay (WST assay). The absorbance of 450nm was measured at 72hr after transient transfection in SNU-601 (A), HSC44Luc (B) and 44As3Luc (C) cells.

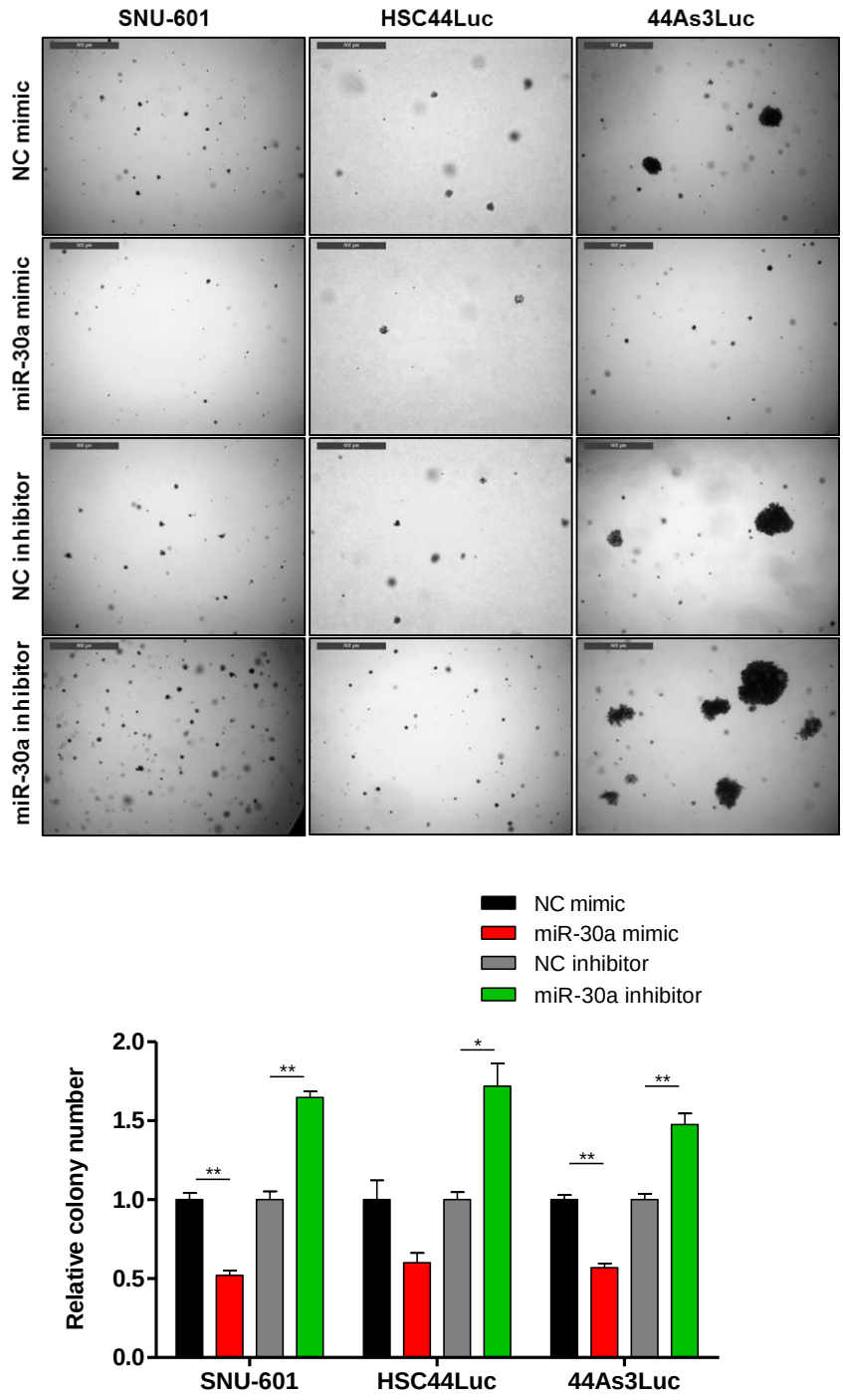


Figure 5. Soft agar colony formation assay

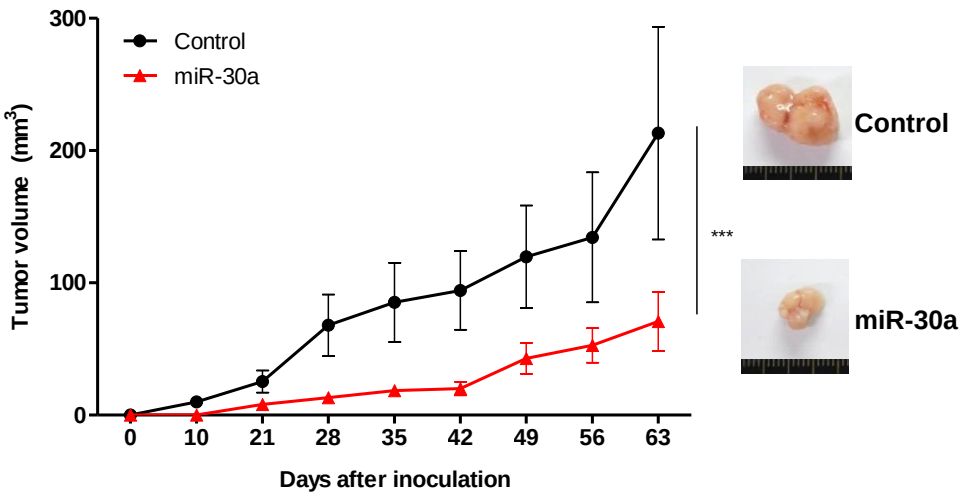


Figure 6. *in vivo* tumorigenesis assay using nude mice. Stable cell lines overexpressing empty vector (NC) or miR-30a were subcutaneously injected into mice (each group; n=8).

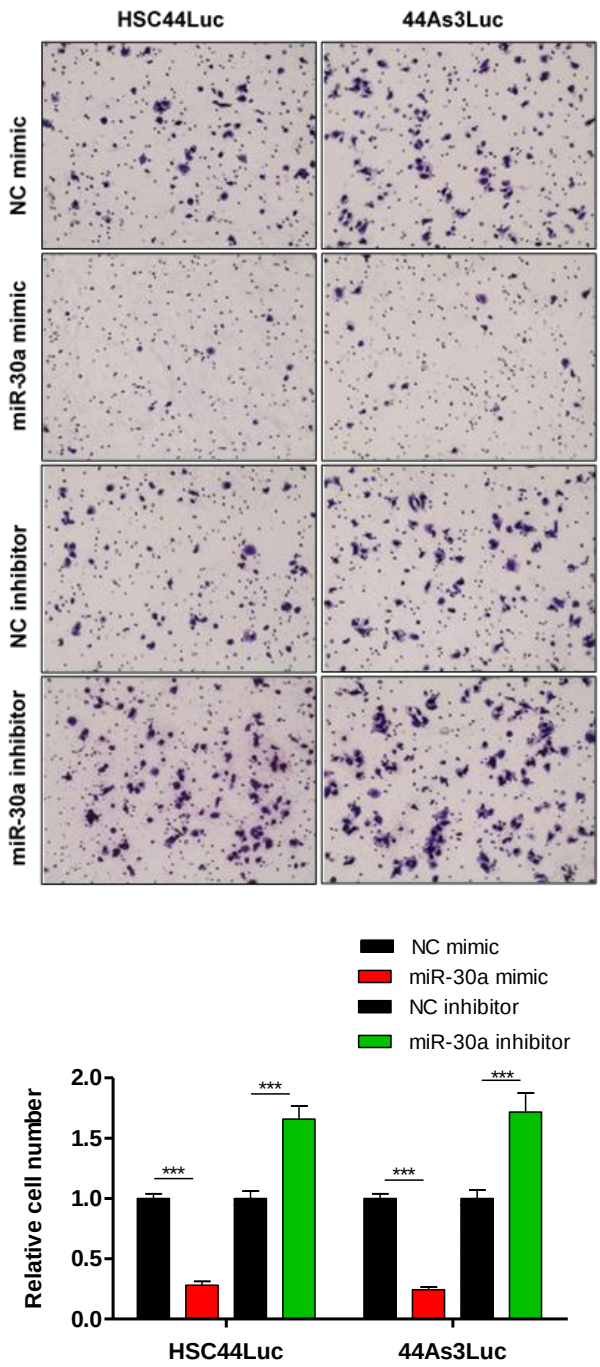


Figure 7. Transwell migration assay

miR-30a affects downregulations of ITGA2, FBXO45, ADAM19 and SEC23A in gastric cancer

Novel miR-30a candidate target genes were revealed by microarray analysis. We identified differentially expressed genes between NC mimic- or miR-30a mimic-treated three gastric cancer cell lines, SNU-601, HSC44Luc, 44As3Luc (Figure 8). We considered that downregulated genes in miR-30a mimic-treated cells may be negatively regulated by miR-30a. Therefore, as shown in Figure 9, we figured out overlapped genes in more than two cell lines. Then, these genes were confirmed using miRNA target gene prediction program such as TargetScan, PITA and miRanda whether these genes are candidate target genes of miR-30a or not. We finally selected ITGA2, FBXO45, ADAM19 and SEC23A as genes downregulated by miR-30a because their expressions were significantly reduced by miR-30a mimic compared to negative control when we validated expressions of these genes in each cell line using qRT-PCR (Figure 10). Thus, miR-30a downregulates ITGA2, FBXO45, ADAM19 and SEC23A in gastric cancer cells.

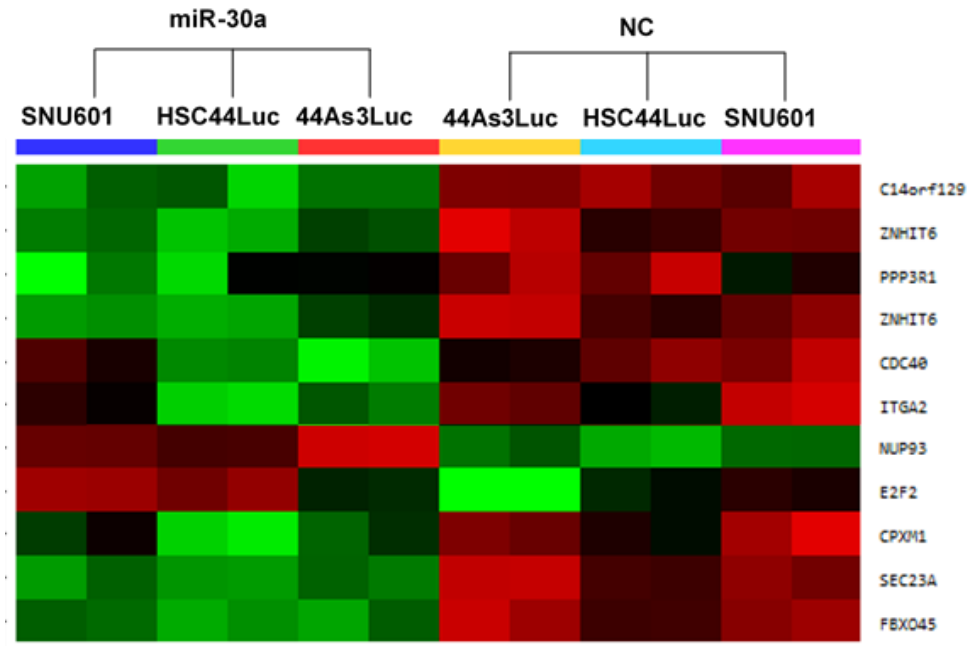


Figure 8. Heat map for differentially expressed genes from microarray data. Negative control mimic (NC) or miR-30a mimic (miR-30a) were treated into SNU-601, HSC44Luc and 44As3Luc cells.

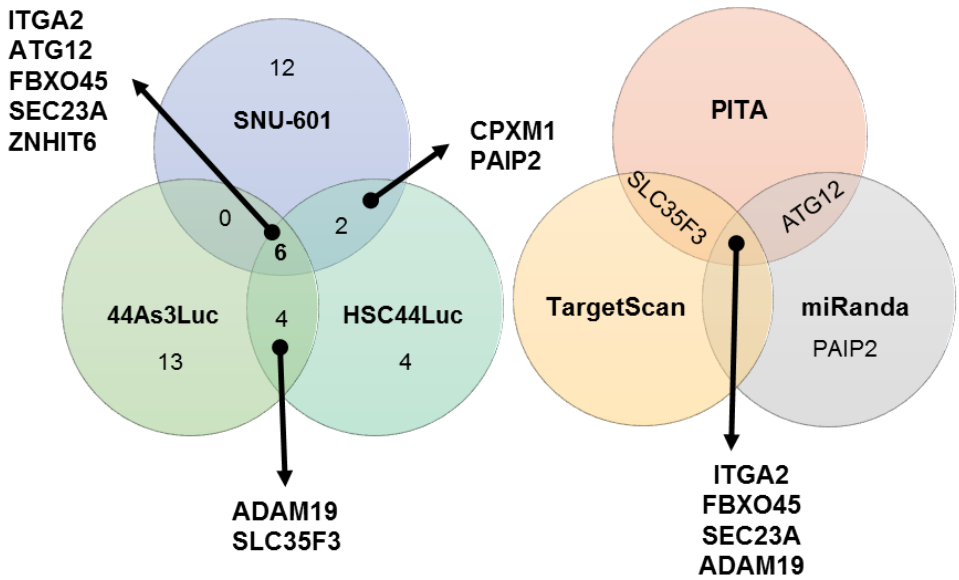


Figure 9. miR-30a candidate target genes. miR-30a candidate target genes which were downregulated in miR-30a mimic-treated cells compared to control from microarray data (left) and which have miR-30a binding sites confirmed using prediction programs (right)

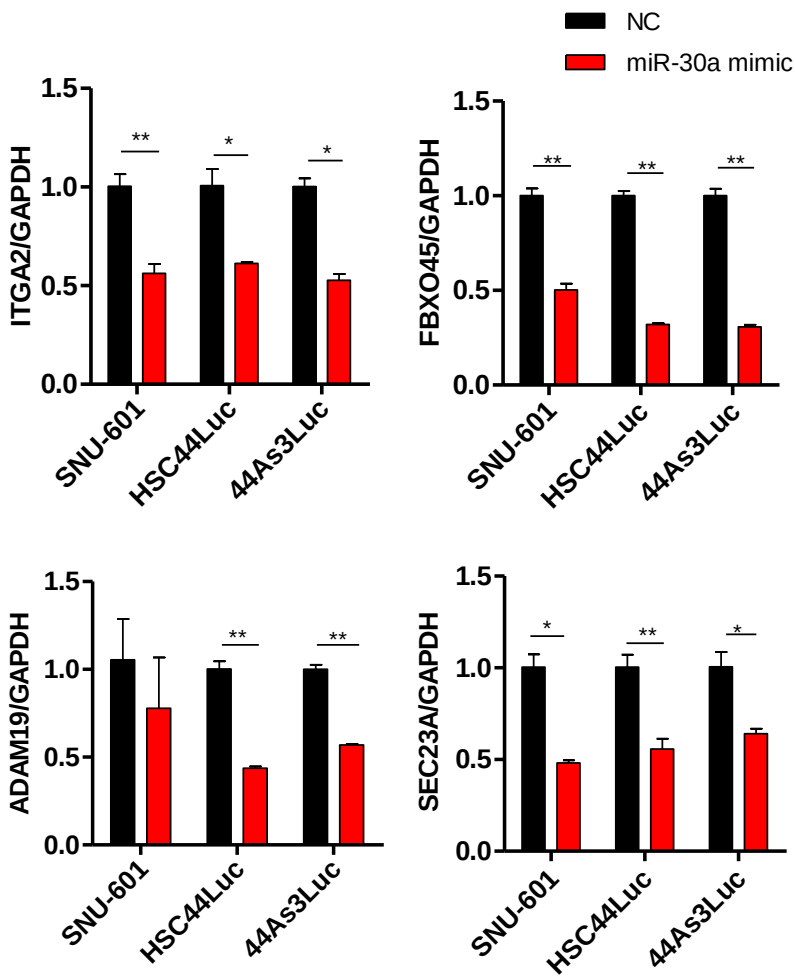


Figure 10. Relative miR-30a expression in miR-30a-mimic treated cells

ITGA2 is directly targeted by miR-30a in gastric cancer

In order to determine associations of each four gene with miR-30a, we screened their expressions in both TCGA cohort and 30 pairs of gastric cancer tissues as the same cohort used for miR-30a expression screening. In TCGA data, ITGA2, FBXO45 and ADAM19 expressions were significantly higher in tumor than in normal (Figure 11A), and expressions of ITGA2 and FBXO45 were inversely correlated with miR-30a expression (Figure 11B). In our cohort, only ITGA2 was significantly upregulated in gastric cancer compared to their matched normal mucosa even though FBXO45, ADAM19 and SEC23A expressions were not different (Figure 12A). Also, it was inversely correlated with miR-30a expression (Figure 12B). Therefore, HEK293T cells were transiently transfected with plasmids containing wild-type of ITGA2 3' UTR or mutant-type of ITGA2 3' UTR regions to confirm whether miR-30a can directly interact with ITGA2. miR-30a binding site of ITGA2 was shown in Figure 13A. When miR-30a mimic was cotransfected, luciferase activity was lesser in cell lysates containing luciferase vector inserted wild-type ITGA2 3' UTR than in negative control. However, miR-30a didn't interact with mutant-type ITGA2 3' UTR (Figure 13B). According to these results, miR-30a can directly target ITGA2 in gastric cancer.

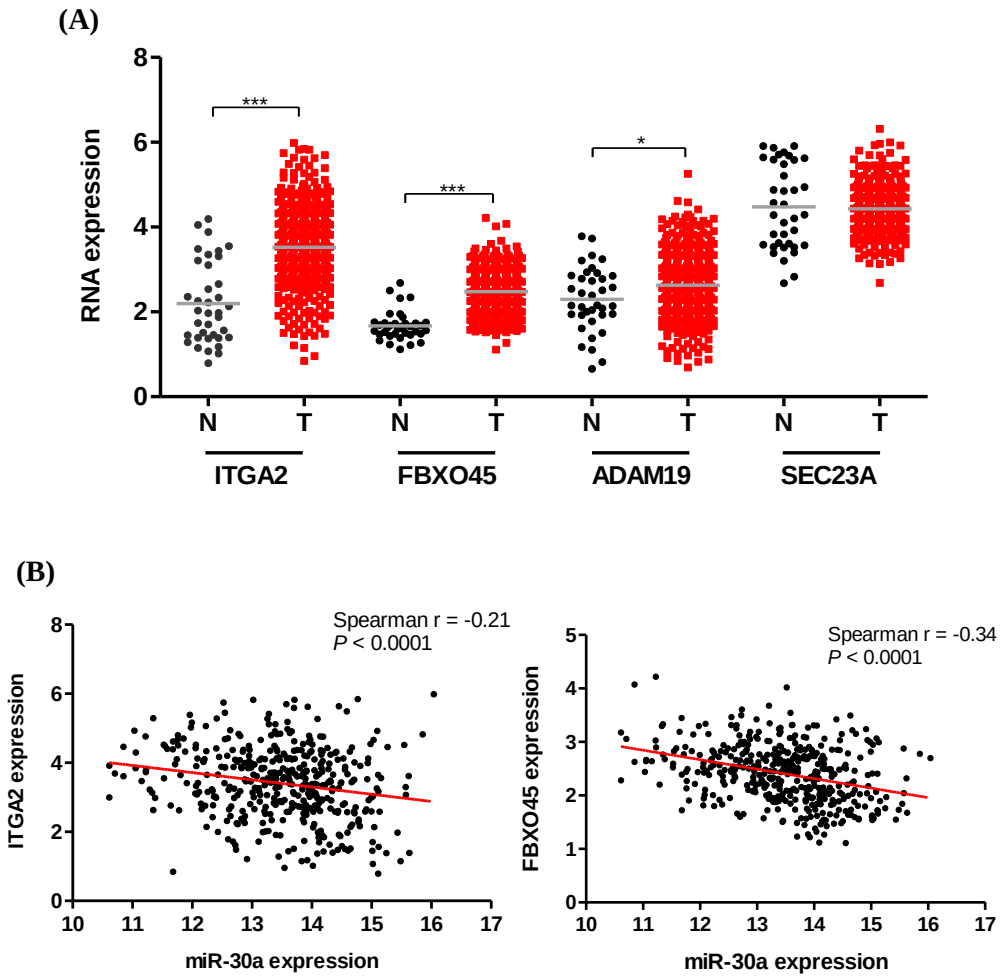


Figure 11. ITGA2, FBXO45, ADAM19 and SEC23A expression analysis using TCGA data. Their expressions between 37 normal (N) and 384 tumor (T) tissues were analyzed using RNA sequencing data of TCGA (A). ITGA2 and FBXO45 were inversely correlated with miR-30a expression (B).

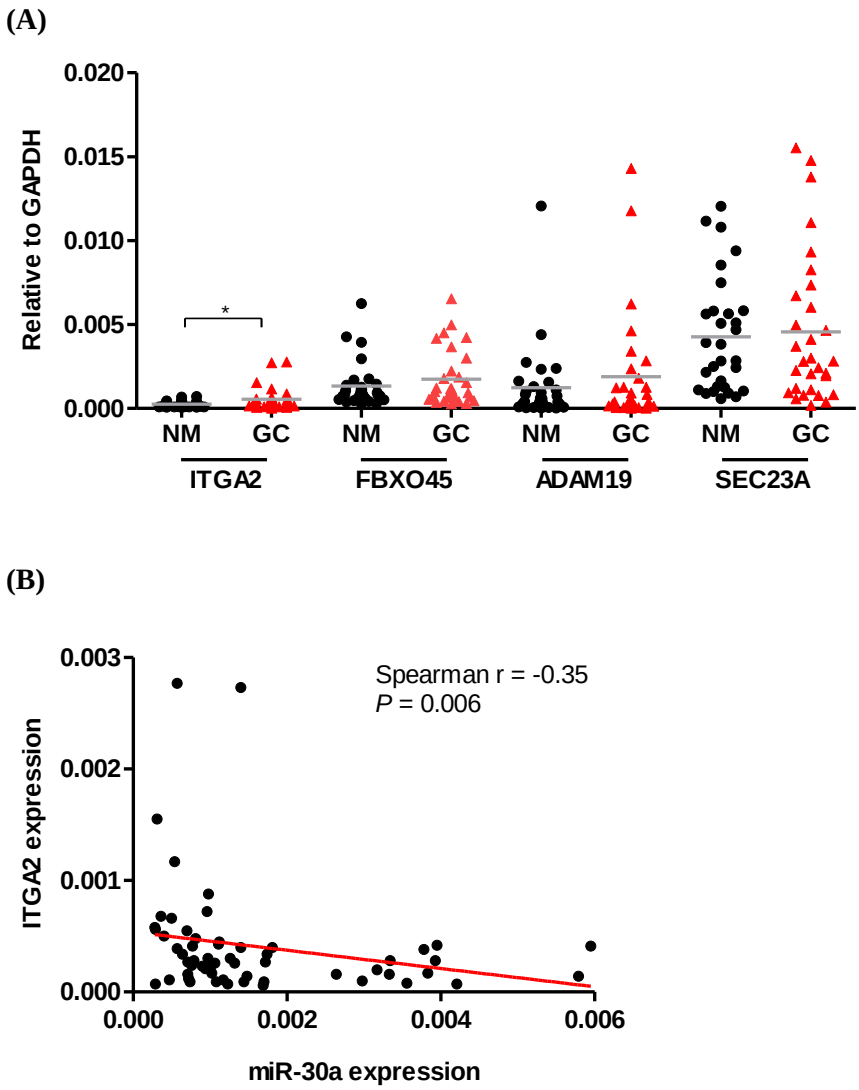


Figure 12. ITGA2, FBXO45, ADAM19 and SEC23A expression analysis using our cohort. Analysis of ITGA2, FBXO45, ADAM19 and SEC23A expressions in 30 pairs of gastric cancer tissues (GC) and adjacent normal mucosa (NM) using qRT-PCR (A) and correlation between miR-30a and ITGA2 expressions (B)

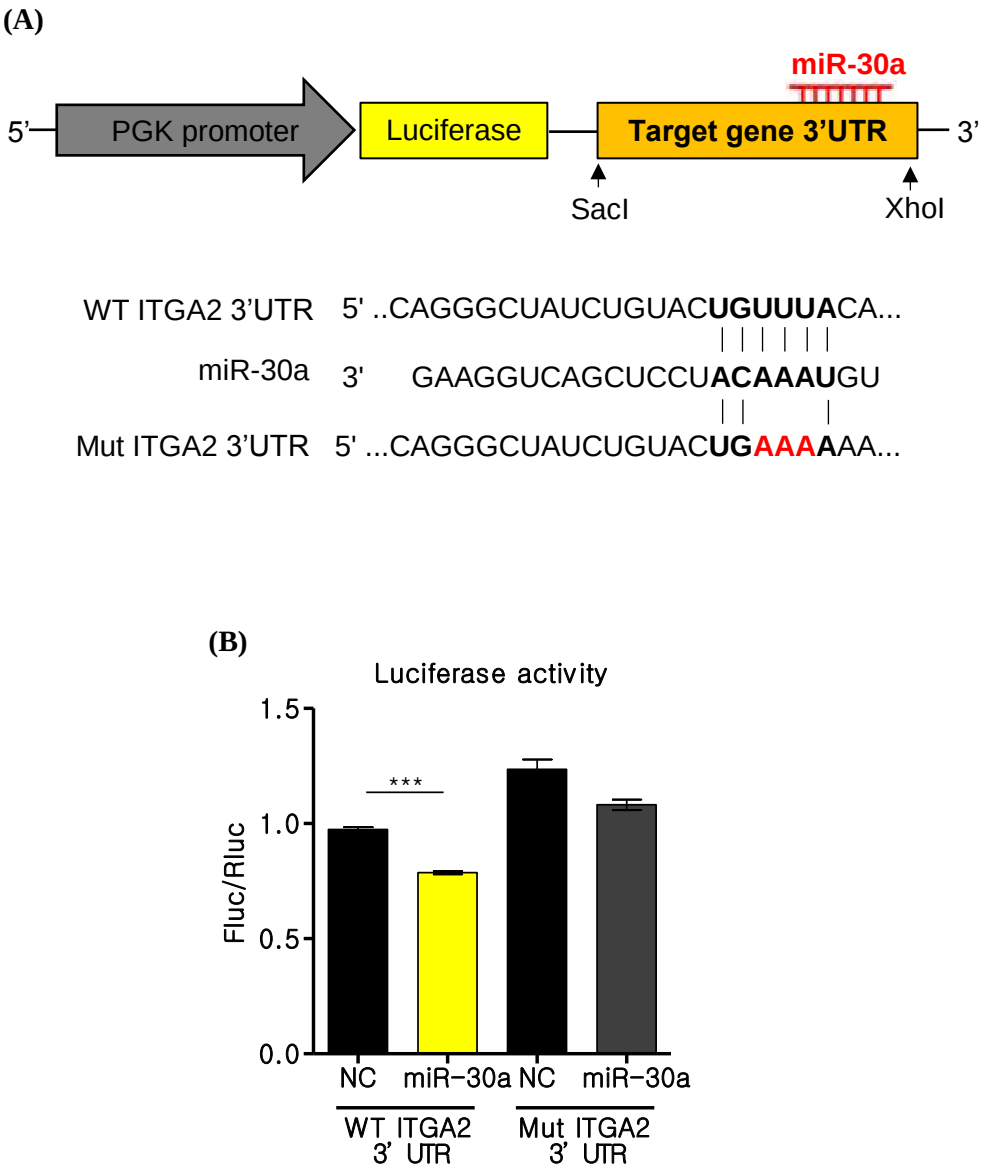


Figure 13. Luciferase reporter assay. Reporter constructs containing the wild type (WT) or the mutant (Mut) of ITGA2 3' UTR regions (A) and result of luciferase reporter assay

ITGA2 knockdown suppresses gastric cancer cell properties

So far, ITGA2 function has been few studied in gastric cancer. Because ITGA2 is a direct target of miR-30a in this study, we treated siRNA for ITGA2 in gastric cancer cell lines to confirm the effects on its suppression in gastric cancer cell growth or motility. As shown in Figure 14A, ITGA2 was differentially expressed in six gastric cancer cell lines. Then, we treated two siRNAs targeting ITGA2 transcript in SNU-601 and 44As3Luc cells and identified the suppression of both mRNA and protein levels that much less than half of negative control (Figure 14B). Cell proliferation was significantly reduced in these two cell lines at 24hr, 48hr and 72hr following siRNA treatments (Figure 15). Also, colony formation ability was quite suppressed in siRNA-treated cells compared to negative control (Figure 16). Since miR-30a regulation affected to cell migration, we also measured migrated cells after si-ITGA2 treatment. As shown in Figure 17, ITGA2 knockdown led to significant reduction of migrated cells compared to control cells. These data revealed that ITGA2 knockdown inhibits proliferative and migratory characteristics of gastric cancer cells.

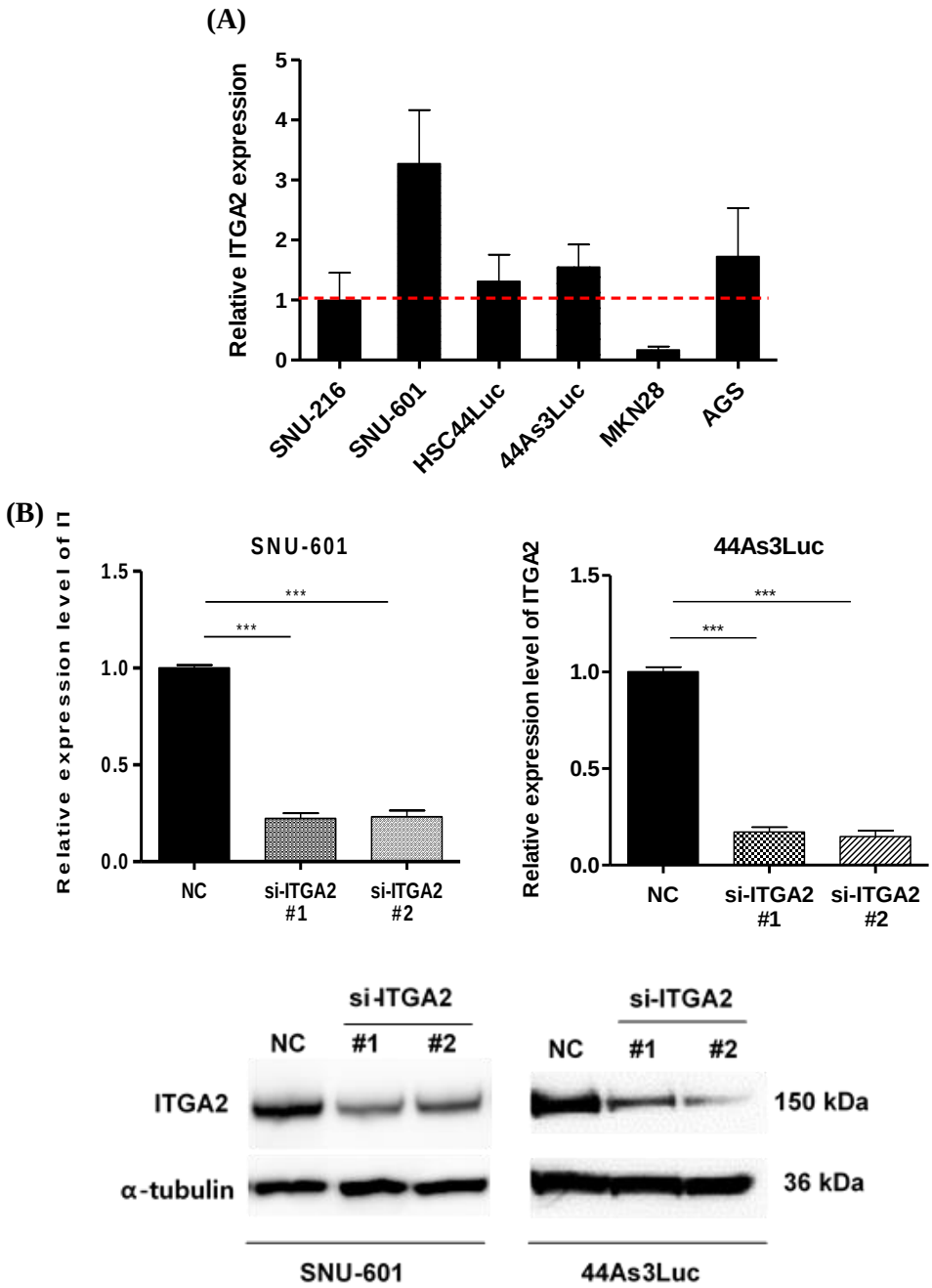


Figure 14. Relative expression level of ITGA2 in gastric cancer cell lines. ITGA2 transcript level in 6 gastric cancer cell lines (A) and its mRNA and protein knockdown by two siRNAs (si-*ITGA2* #1 and #2) in SNU-601 and 44As3Luc cells compared to negative control siRNA (NC) (B)

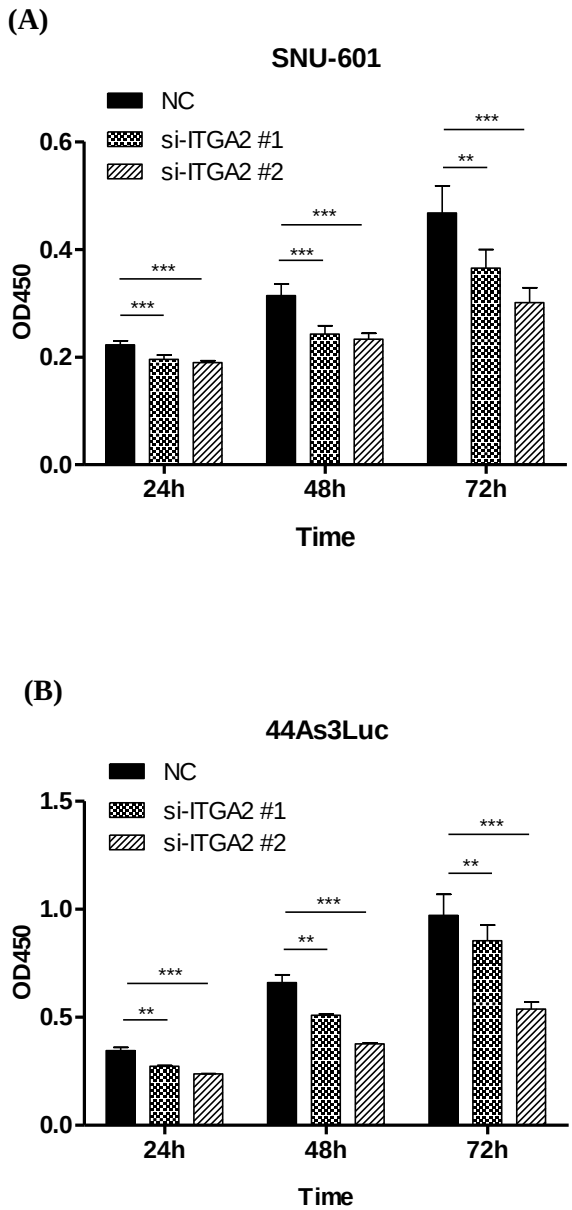


Figure 15. Cell proliferation assay (WST-assay). The absorbance at 450nm was measured at 24hr, 48hr and 72hr after siRNA treatment in SNU-601 (A) and 44As3Luc (B)

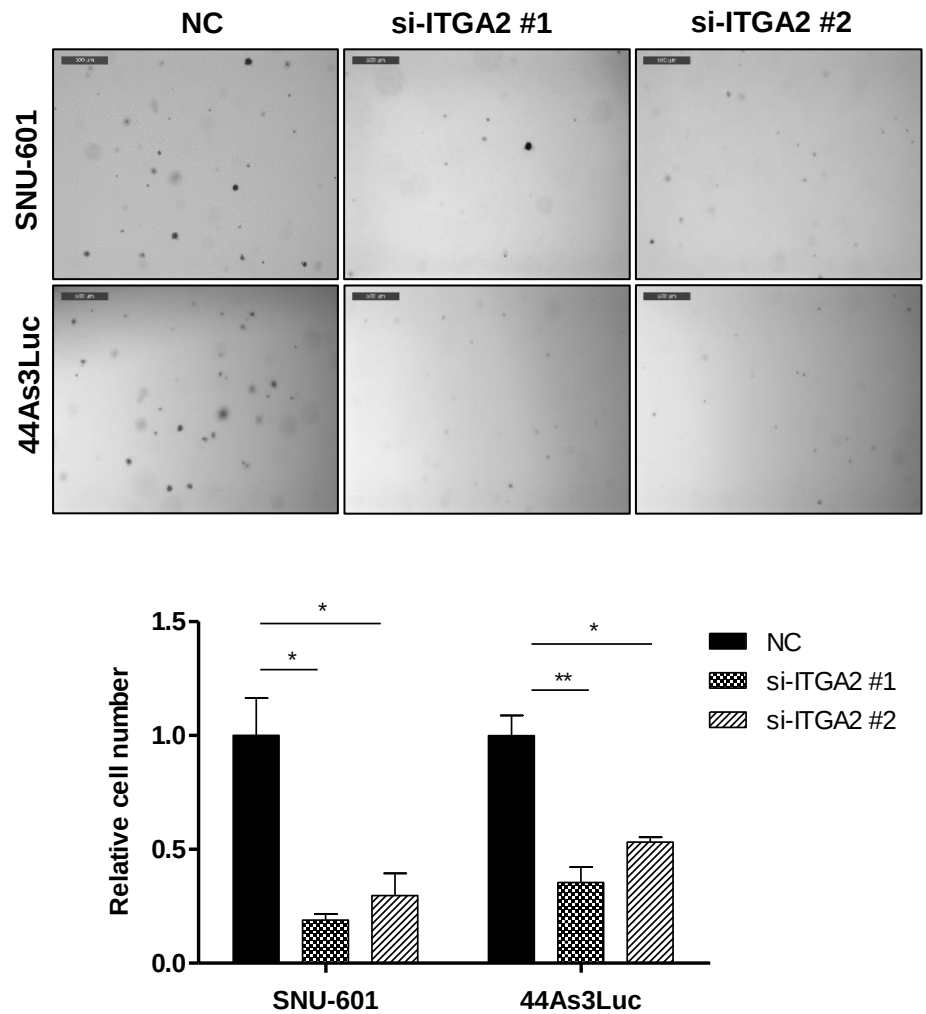


Figure 16. Soft agar colony formation assay. The anchorage independent cell growth was measured by counting relative colony numbers.

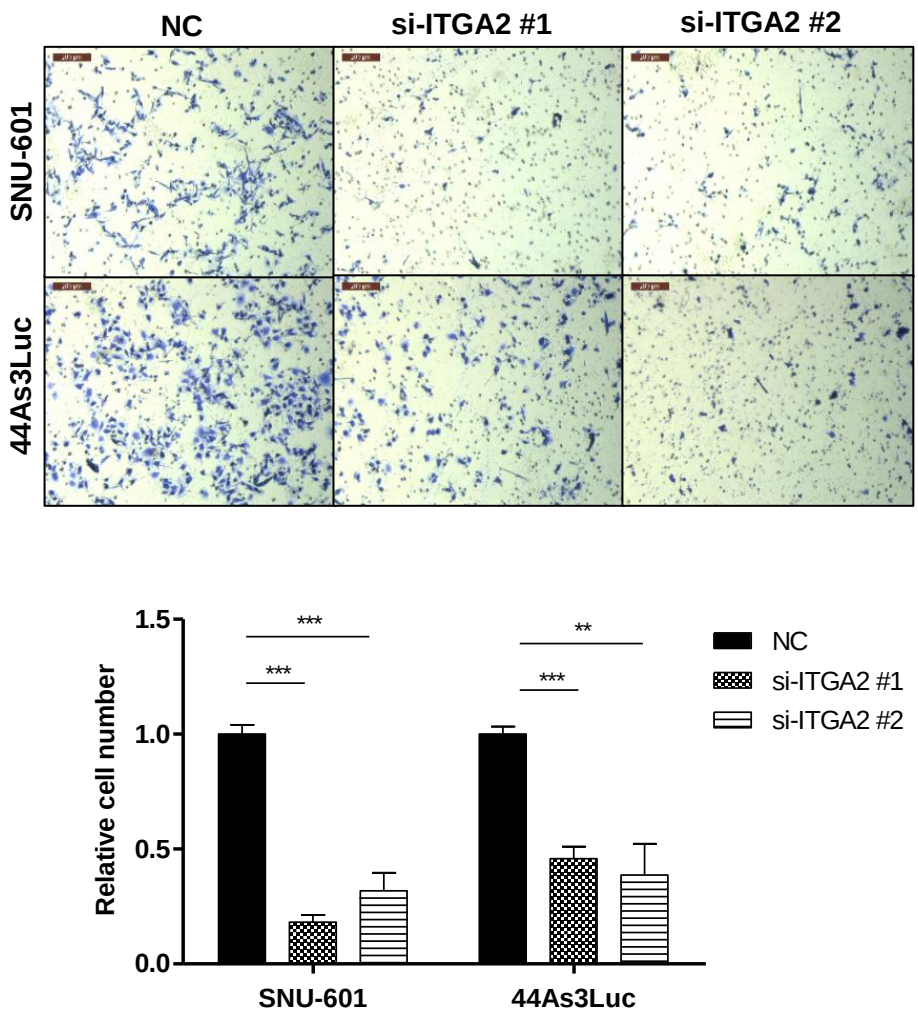


Figure 17. Transwell migration assay. Relative migrated cells were counted compared to negative control (NC) after siRNA treatment

Clinicopathologic and prognostic relevances of miR-30a-ITGA2 expression in gastric cancer patients

To investigate whether miR-30a and ITGA2 expressions can be biomarkers for clinicopathological parameters in gastric cancer, we first analyzed clinical information from NCBI GEO data and TCGA cohort. In NCBI GEO data, the significant downregulation of miR-30a was observed in *H. pylori*-infected gastric cancer. In contrast, ITGA2 was significantly upregulated in *H. pylori*-infected intestinal metaplasia compared to *H. pylori*-not infected normal (Figure 18). We additionally analyzed both miR-30a and ITGA2 expressionis according to MSI, *H. pylori* infection, histological type, lymph node metastasis and TNM stage from TCGA data. As shown in Figure 19A, miR-30a was more downregulated in MSI-high compared with both microsatellite stability (MSS) and MSI-low types, and the opposite result was obtained from ITGA2. There were inverse correlations between miR-30a and ITGA2 in each MSS and MSI-high type (Figure 19B). Also, we observed significantly lower mean value of miR-30a in intestinal type gastric cancer than in diffuse type gastric cancer, while ITGA2 was upregulated in intestinal type (Figure 20A). They were also inversely correlated each other in each histological type (Figure 20B). In our cohort, miR-30a expression was not different between intestinal and diffuse type, but ITGA2 was significantly upregulated in intestinal type (Figure 21A). It also had inverse correlation with miR-30a in intesitnal type (Figure 21B).

Next, we conducted pathological examination using tissue microarray (TMA) to evaluate associations between ITGA2 and clinical implications. In the result of TMA, membranous or cytoplasmic expressions of ITGA2 were identified, and each

expression was individually scored in the range of negative (score 0) to strong positive (score 3) as shown in Figure 22. We analyzed each score according to clinical features such as TNM stage, histological type, differentiation status and patient survival. Cytoplasmic ITGA2 was associated with only lauren classification, but there were no significant diffences between all of clinicopathological features and membranous ITGA2 expression. Stronger staining intensities of cytoplasmic ITGA2 tended to be included more in intestinal type gastric cancer than in diffuse type gastric cancer, and the difference was statistically significant (Figure 23). These data indicated that miR-30a can be a biomarker for *H. pylori* infection and revealed that miR-30a-ITGA2 axis is useful to predict both MSI and lauren classification in gastric cancer. Especially in protein level, ITGA2 expression in cytoplasm could be a marker for lauren classification.

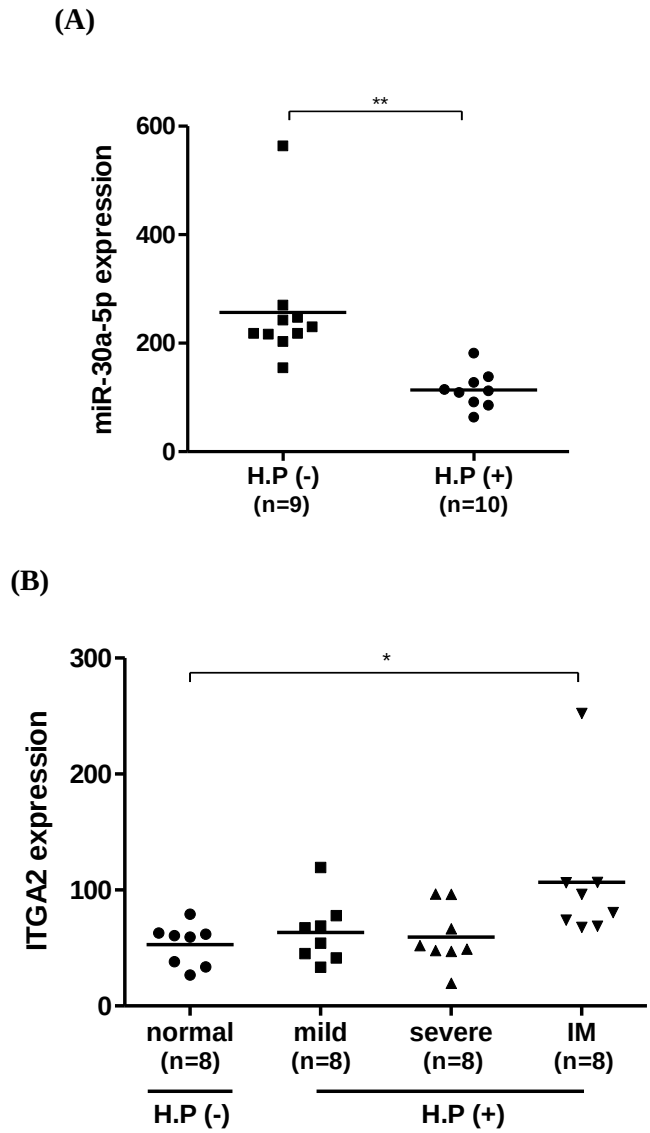


Figure 18. miR-30a-5p and ITGA2 expressions according to *H. pylori* infection. miR-30a-5p expression in *H. pylori*-infected (H.P (+), n=9) or -not infected (H.P (-), n=10) gastric cancer analyzed from GSE19769 (A) and ITGA2 expression in H.P (-) normal (n=8), mild gastritis (n=8), severe gastritis (n=8) and intestinal metaplasia (IM, n=8) analyzed from GSE60427 data (B) of NCBI GEO

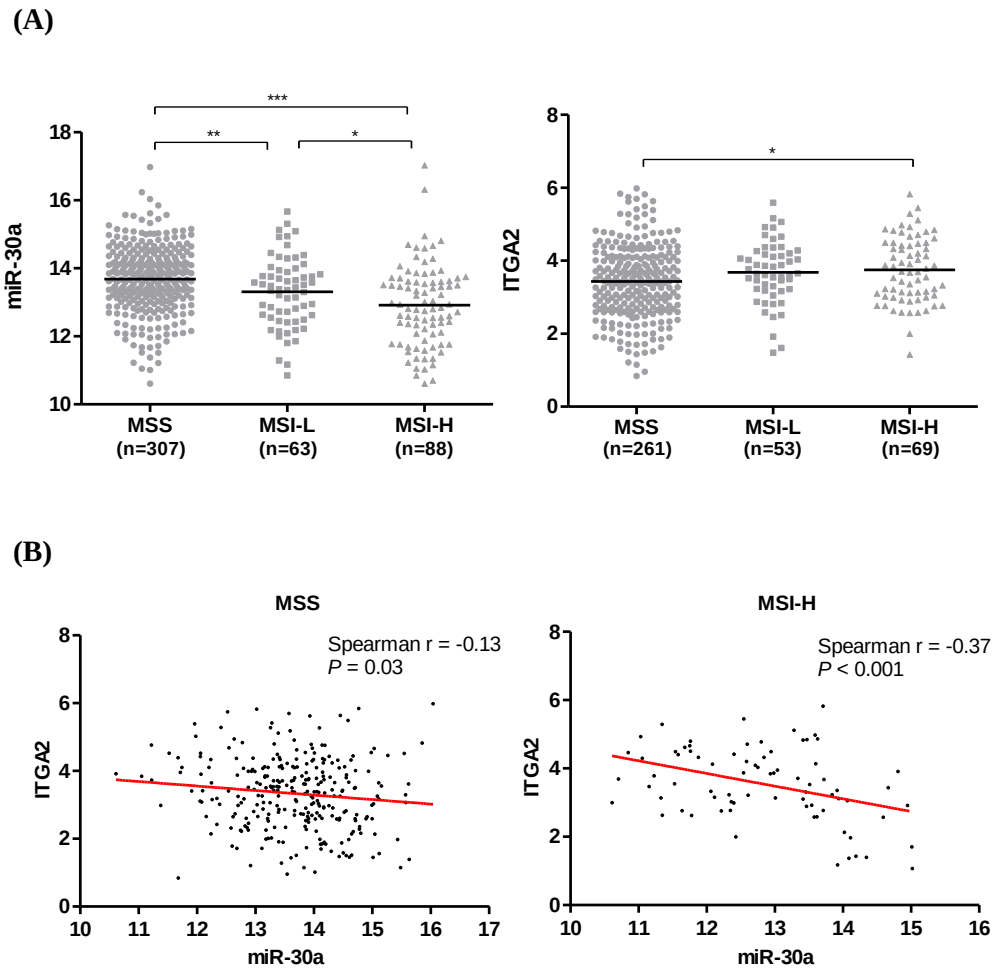


Figure 19. miR-30a and ITGA2 expression analysis (A) and correlation analysis (B) according to MSI. Expression and correlation analyses were performed according to MSS (n=307), MSI-low (MSI-L, n=63) and MSI-high (MSI-H, n=88) using TCGA data.

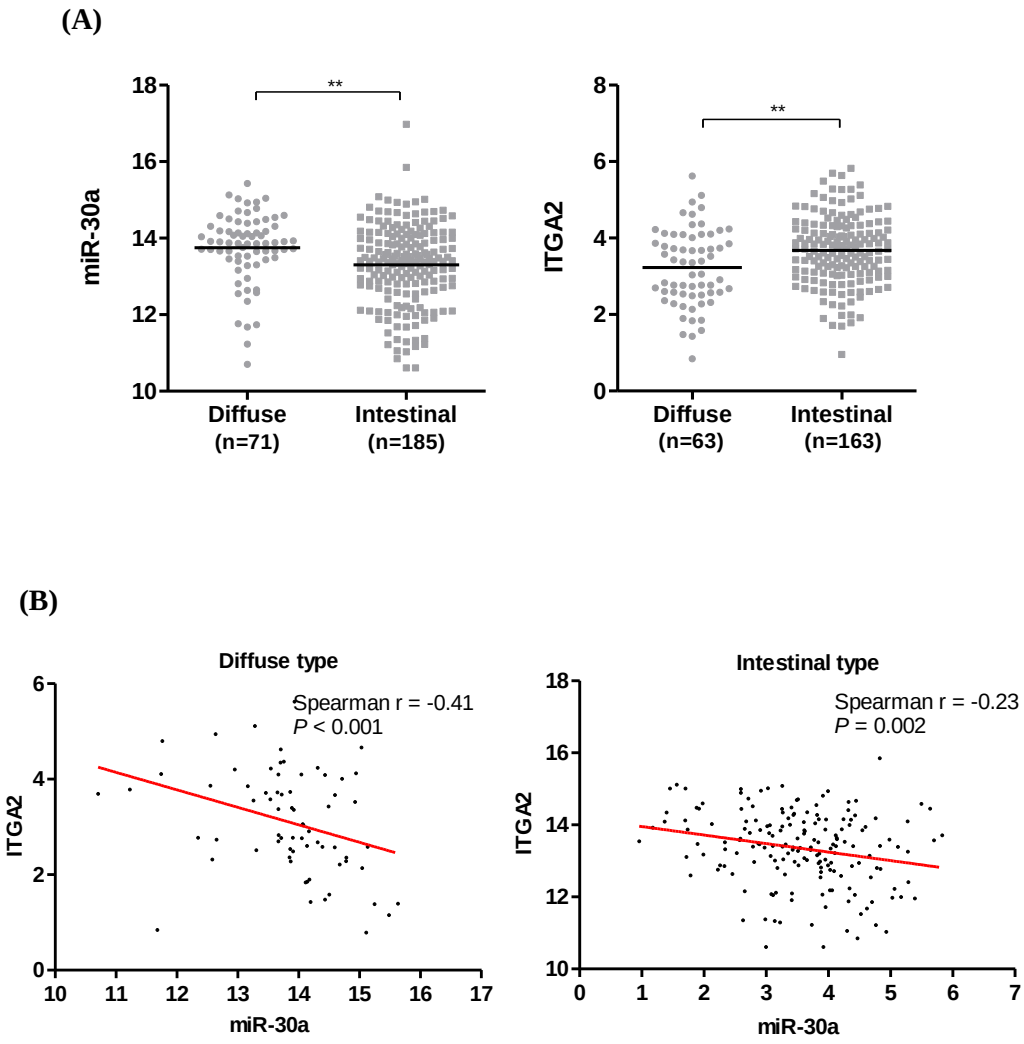


Figure 20. miR-30a and ITGA2 expression analysis (A) and correlation analysis (B) according to Lauren classification using TCGA data. Expression data according to diffuse type (n=71) and intestinal type (n=185) gastric cancer from TCGA.

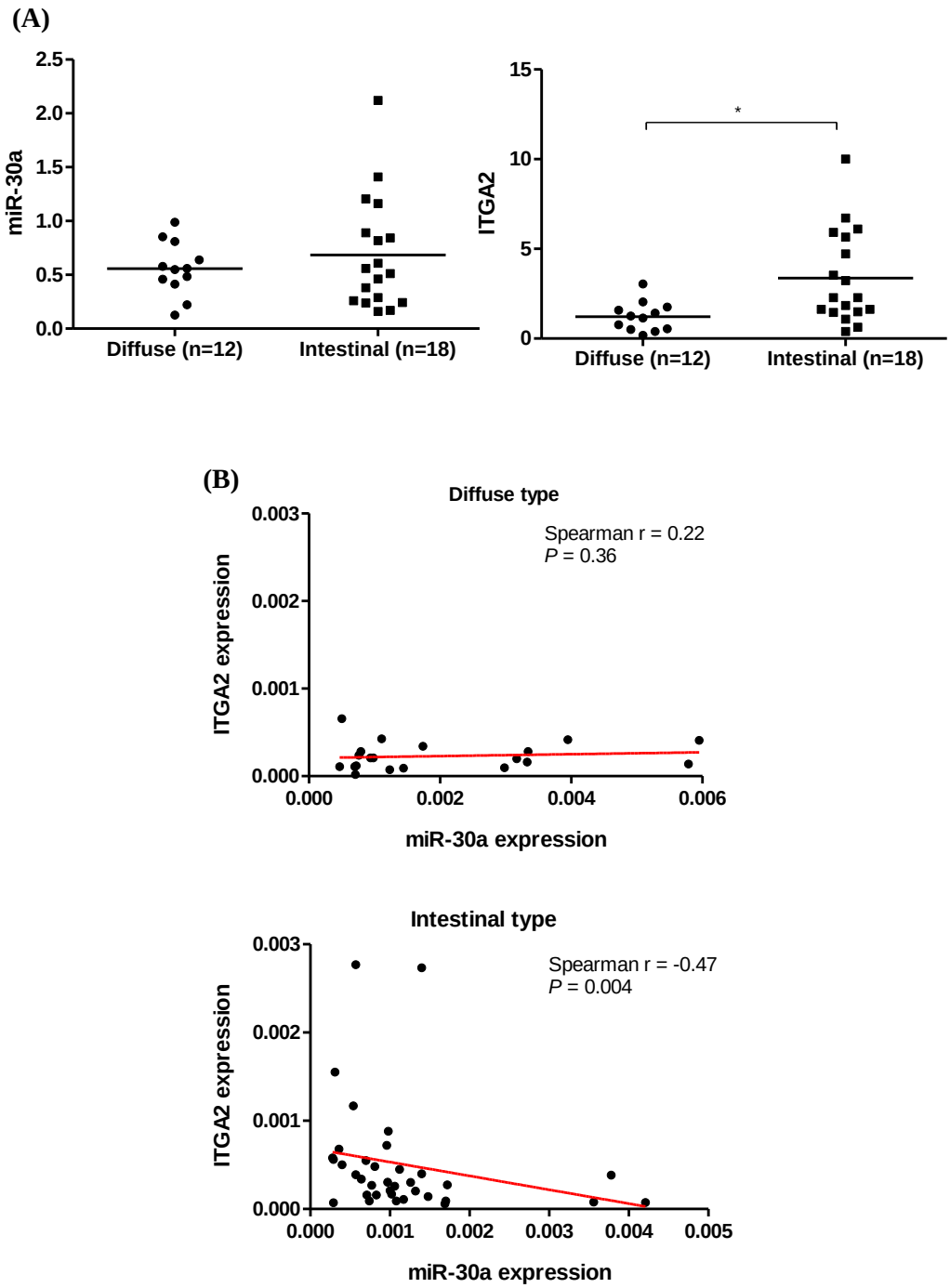


Figure 21. miR-30a and ITGA2 expression analysis (A) and correlation analysis (B) according to Lauren classification using our cohort. Analyses according to diffuse type (n=12) and intestinal type (n=18) gastric cancer from our cohort.

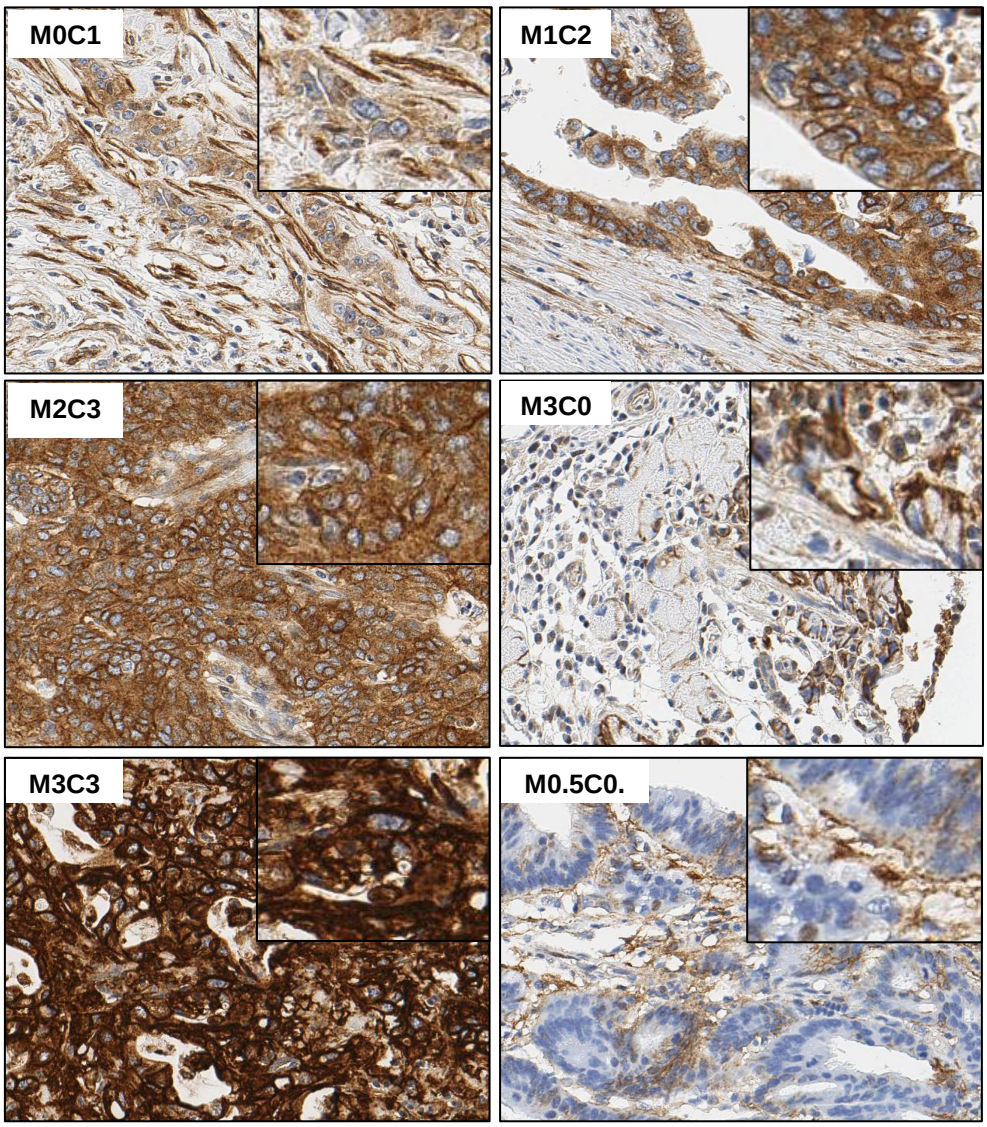


Figure 22. The representative results of ITGA2 immunohistochemical staining. ITGA2 staining intensity was scored individually in cellular membrane (M) or cytoplasm (C)

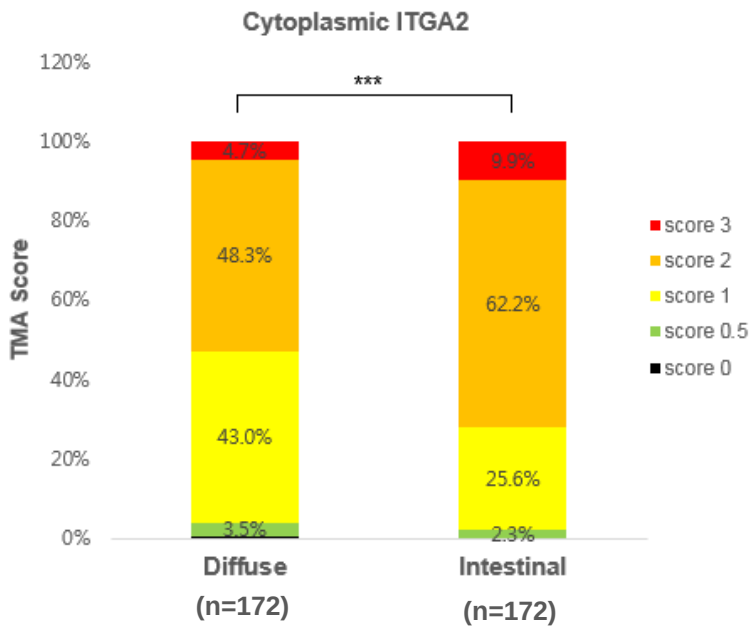


Figure 23. ITGA2 cytoplasmic expression according to Lauren classification. Intestinal type gastric cancer (n=172) had higher mean value of TMA score than diffuse type gastric cancer (n=172)

Part II.

Long interspersed nuclear element-1 (LINE-1), one of miR-30a regulators, is associated with clinicopathological features in gastric cancer

Purpose of this study

We hypothesized that LINE-1 would be a prognostic marker in different tissue types of gastric cancer. Therefore, the aim of this study is to validate LINE-1 methylation pattern in two gastric cancer tissue types, formalin-fixed paraffin-embedded (FFPE) and frozen tissues, and to evaluate whether LINE-1 methylation can be an epigenetic marker for several clinicopathological features in each tissue type of gastric cancer.

Material and method

Tissue specimens

Forty-one pairs of primary gastric cancer tissues and corresponding normal mucosa were immediately frozen in liquid nitrogen without fixation following gastrectomy, and tumor cellularity was confirmed by a pathologist. Twenty-five tumor tissues were fixed with formalin and embedded in paraffin, and only tumor area was confirmed by a pathologist. The clinicopathological characteristics of total 66 patients are indicated in Table 4. LINE-1 methylation status was analyzed according to gender, age, WHO classification, Lauren classification, tumor location, lymphatic invasion, venous invasion, perineural invasion, TNM stage and MSI. Tissue specimens and written informed consent from all patients were obtained from Seoul National University Hospital, Korea. This study was approved by the institutional review boards (IRB) of Seoul National University Hospital (IRB No. 1501-086-642).

Table 4. Clinicopathological features of 66 gastric cancer patients

Characteristic	Gastric cancer (n=66)	Percentage
Gender		
Male	42	63.64%
Female	24	36.36%
Age, years		
≤ 59	31	46.97%
> 59	35	53.03%
T classification		
T1	12	18.18%
T2	14	21.21%
T3	21	31.82%
T4	19	28.79%
N classification		
N0	28	42.42%
N1	7	10.61%
N2	8	12.12%
N3	23	34.85%
Distance metastasis		
Absent	62	93.94%
Present	4	6.06%
TNM stage		
I	0	0.00%
II	49	74.24%
III	13	19.70%
IV	4	6.06%
Lauren Classification		
Intestinal	28	42.42%
Diffuse	27	40.91%
Mixed	2	3.03%
Unknown	9	13.64%
WHO classification		
Differentiated	24	36.36%
Undifferentiated	35	53.03%
Unknown	7	10.61%

Tumor location		
Upper	16	24.24%
Middle	13	19.70%
Lower	36	54.54%
Entire	1	1.52%
Lymphatic invasion		
Not identified	22	33.33%
Present	25	37.88%
Unknown	19	28.79%
Venous invasion		
Not identified	40	60.61%
Present	7	10.61%
Unknown	19	28.79%
Perineural invasion		
Not identified	28	42.42%
Present	19	28.79%
Unknown	19	28.79%

DNA extraction and sodium bisulfite modification

Genomic DNA (gDNA) was extracted from frozen tissues using the QuickGene DNA tissue kit S with QG-Mini80 (Kurabo, Japan) and from FFPE tissues using the QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's protocols. Sodium bisulfite modification of 600 ng DNA was performed using the EpiTect Bisulfite Kit (Qiagen, Germany) according to the manufacturer's instructions. The reaction was set under following conditions: 5 min at 95℃, 25 min at 60℃, 5 min at 95℃, 85 min at 60℃, 5 min at 95℃, 175 min at 60℃, and overnight at 20℃. The final products were stored at -20℃ until further use.

Quantitative bisulfite pyrosequencing for LINE-1 and methylation analysis

Bisulfite pyrosequencing for LINE-1 was performed as previously described [55]. PCR reaction was conducted in a volume of 20 μl, with ≥20 ng bisulfite modified gDNA, PCR premixture (Enzynomics, Korea), 10 pmol/μl forward and biotinylated reverse primers. The reaction was performed according to the following conditions: 10 min at 95℃; followed by 45 cycles at 95℃ for 30 s, 53℃ for 30 s, and 72℃ for 30 s; and 5 min at 72℃. The 2 μl of PCR products were loaded by electrophoresis using a 2% agarose gel with ethidium bromide. A single-stranded DNA template was prepared from 16-18 μl of the biotinylated PCR product using streptavidin Sepharose® HP beads (Amersham Biosciences, Sweden) following the PSQ 96 sample preparation guide. Sequencing was performed on a PyroMark ID system with a Pyro Gold reagents kit (Qiagen) according to the manufacturer's instructions without further optimization, and 15 pmol/μl sequencing primer was used for

analysis. The primers are described in Table 5. The average of LINE-1 methylation level was calculated from the methylation percentage at 4 CpG sites. In Figure 24, four CpG sites of LINE-1 that we analysed in this study were indicated. Patients were grouped according to each clinicopathological parameter to compare LINE-1 methylation levels. In all clinicopathological parameters, the average methylation percentage of all CpG sites and each four CpG site were analysed.

Statistical analysis

To compare the LINE-1 methylation levels in frozen 41 pairs of gastric cancer and matched normal mucosa, a paired t-test was performed. An unpaired t-test was used to compare LINE-1 methylation between each classified groups according to clinicopathological parameters. The ANOVA test was used in analysis of the tumor location and TNM Stage. Statistically significant difference was considered at p-value < 0.05. We used GraphPad Prism V5.0 (GraphPad Software, USA) for statistical analyses.

Table 5. Primers for LINE-1 methylation analysis

Primer	**Sequence (5' → 3')**
Forward primer for PCR	TTTTGAGTTAGGTGTGGGATATA
Reverse primer for PCR	Biotin-AAAATCAAAAAATTCCCTTTC
Sequencing primer	AGTTAGGTGTGGGATATAGT

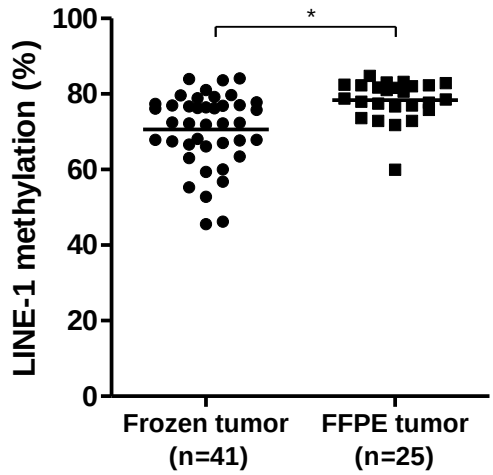
Figure 24. Four CpG sites of LINE-1 for methylation analysis

Result

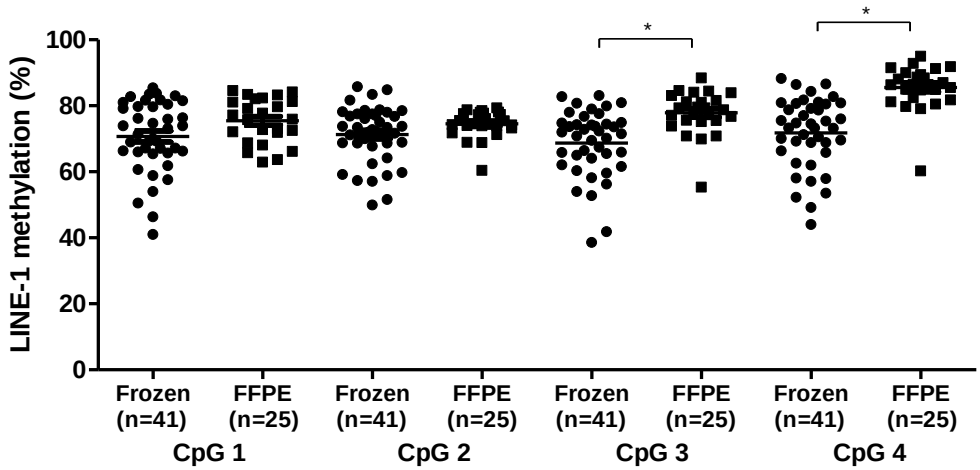
Validation of LINE-1 methylation patterns in frozen and FFPE tissues of gastric cancer

As previously reported, LINE-1 methylation percentages are different between fresh tissue and FFPE tissue [54]. Accordingly, we validated its methylation levels at four CpG sites of the LINE-1 promoter using our frozen and FFPE tissue samples. The average methylation level of LINE-1 was significantly different between 41 frozen tumor tissues and 25 FFPE tumor tissues ($p < 0.001$, Figure 25A). When we compared methylation level at each CpG site, LINE-1 was significantly hypermethylated at CpG3 and CpG4 of FFPE tumor tissues compared to those of frozen tumor tissues (CpG3; $p < 0.001$, CpG4; $p < 0.001$, Figure 25B). In each tissue type, LINE-1 methylation levels were different at each CpG site (Figure 25C and 25D). These results verified LINE-1 methylation status differed between each CpG site as well as each type of tissue sample.

(A)


LINE-1 methylation (%)
100
80
60
40
20
0
*
Frozen tumor (n=41)
FFPE tumor (n=25)

(B)


LINE-1 methylation (%)
100
80
60
40
20
0
*
*
Frozen (n=41)
FFPE (n=25)
CpG 1
CpG 2
CpG 3
CpG 4

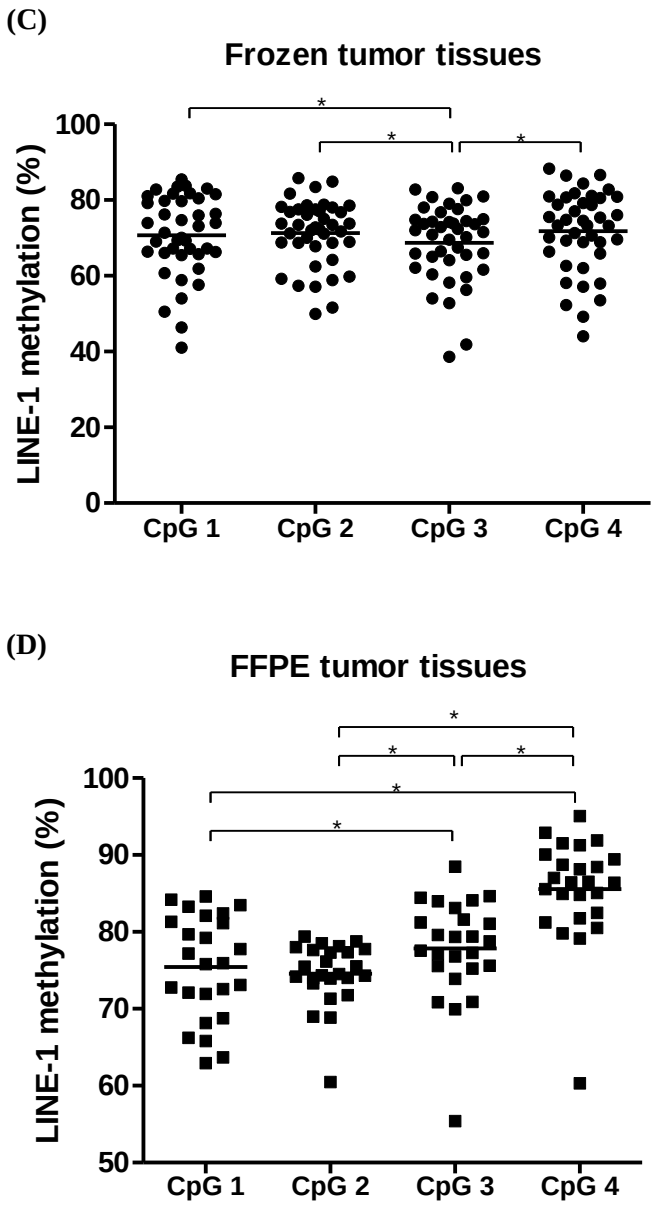


Figure 25. LINE-1 methylation levels in frozen and FFPE tumor tissues. The average methylation level of all CpG sites (A), the methylation level at each CpG site (B) between frozen and FFPE tumor tissues and LINE-1 methylation level at each CpG site in frozen tumor tissues (C) and in FFPE tissues (D)

Analysis of LINE-1 methylation and clinicopathological characteristics using frozen tissues

To validate whether LINE-1 hypomethylation is shown in our gastric cancer tissue samples before we analyze association between LINE-1 methylation and clinicopathological parameters, 41 pairs of gastric cancer tissues and corresponding normal mucosa were used. As shown in Figure 26, LINE-1 was significantly hypomethylated in gastric cancer compared to normal mucosa ($p < 0.001$). Next, we compared LINE-1 methylation levels according to age, gender, WHO classification, Lauren classification, tumor location, lymphatic invasion, perineural invasion, TNM stage and MSI. As shown in Figure 27, LINE-1 was hypomethylated in the male gender ($p = 0.004$), differentiated gastric cancer ($p = 0.016$), the presence of lymphatic invasion ($p = 0.023$) and venous invasion ($p = 0.016$). Therefore, we additionally analyzed the methylation level at each CpG site. In gender, differentiation status, lymphatic invasion and venous invasion, LINE-1 methylation was also significantly associated with each variable at all CpG site (Table 6).

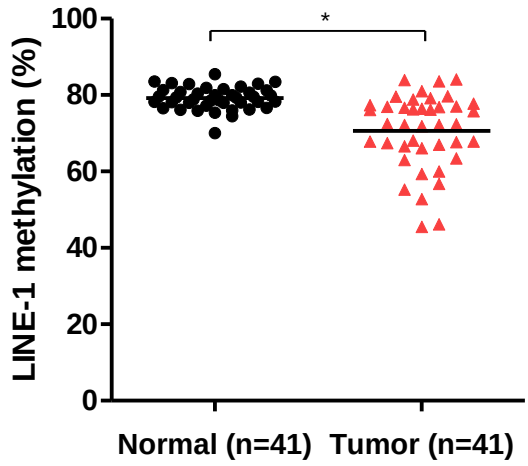


Figure 26. LINE-1 hypomethylation in gastric cancer

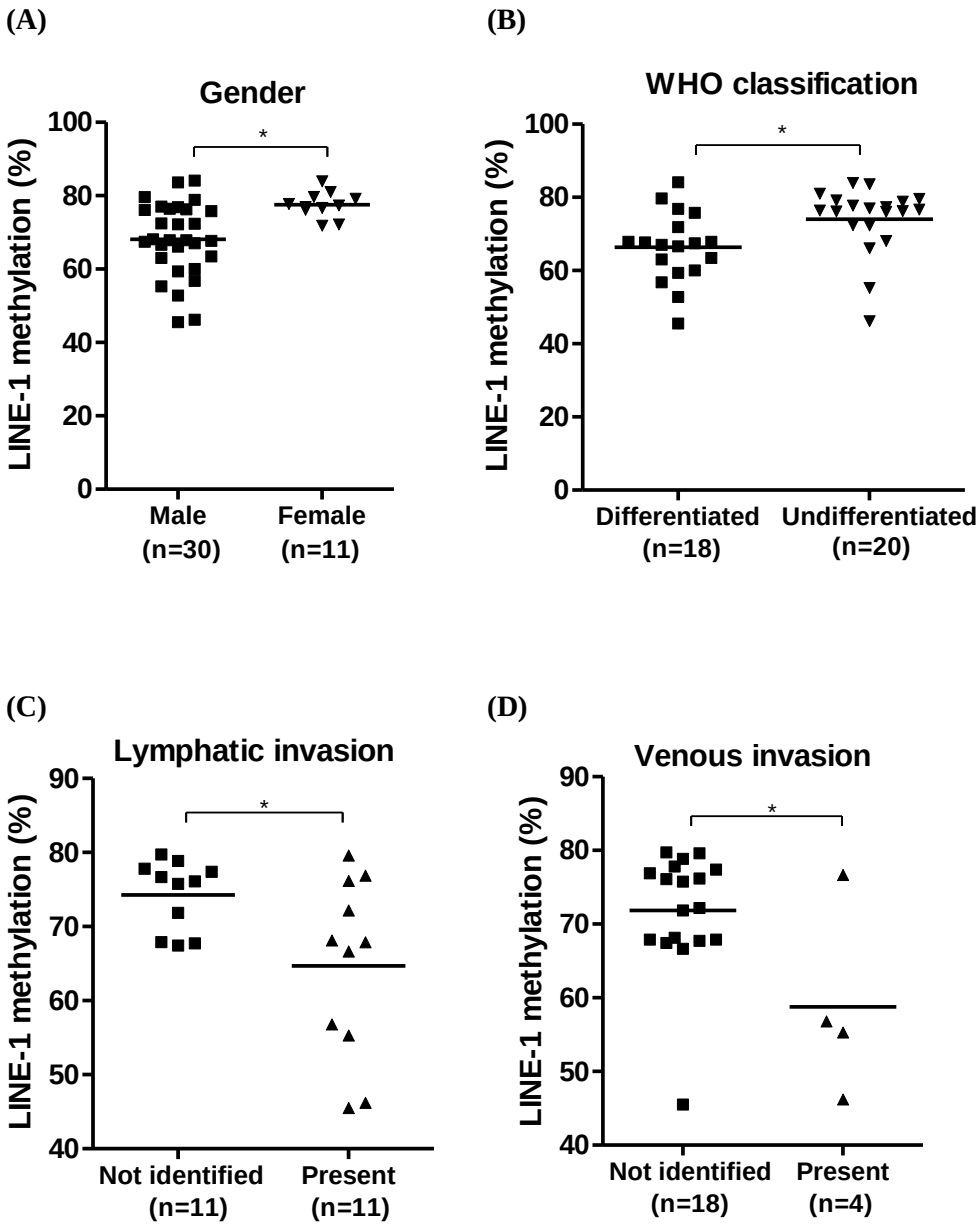


Figure 27. LINE-1 methylation levels according to gender (A), differentiation status (B), lymphatic invasion (C) and venous invasion (D)

Table 6. The methylation level of LINE-1 at four CpG site according to clinicopathological characteristics of frozen tumor tissues

Characteristic	No	LINE-1 methylation level (%)							
		CpG 1	*P* value	CpG 2	*P* value	CpG 3	*P* value	CpG 4	*P* value
Type									
Normal mucosa	41	81.61	<0.001	78.27	<0.001	76.32	<0.001	80.48	<0.001
Gastric cancer	41	70.73		71.32		68.74		71.76	
Age									
≤61	18	70.82	0.965	73.48	0.157	71.12	0.189	74.13	0.215
>61	23	70.66		69.62		66.88		69.90	
Gender									
Male	30	68.42	0.021	69.19	0.007	65.69	<0.001	69.12	0.008
Female	11	77.02		77.12		77.07		78.95	
WHO classification									
Differentiated	18	66.32	0.017	67.38	0.012	64.53	0.036	67.10	0.020
Undifferentiated	20	74.77		74.53		71.53		75.34	
Lauren classification									
Intestinal	20	67.18	0.199	68.40	0.252	65.25	0.211	68.14	0.214
Diffuse	12	72.53		71.97		70.02		73.12	
Tumor location									
Upper	11	69.59	0.887[a]	68.70	0.295[a]	65.29	0.296[a]	68.12	0.207[a]
Middle	8	69.85		75.02		72.80		77.04	
Lower	21	71.40		70.93		68.88		71.31	
Lymphatic invasion									
Not identified	11	75.53	0.041	74.28	0.020	72.00	0.020	75.35	0.029

Present	11	65.28		66.17		61.65		65.60	
Venous invasion									
Not identified	18	72.86	0.037	72.20	0.016	69.34	0.016	73.06	0.012
Present	4	59.36		61.36		55.50		58.82	
Perineural invasion									
Not identified	16	70.90	0.757	70.87	0.570	67.35	0.716	71.11	0.659
Present	6	69.07		68.51		65.41		68.77	
TNM stage									
II	26	70.92	0.922[a]	70.59	0.535[a]	67.58	0.487[a]	71.01	0.532[a]
III	13	69.98		71.79		70.01		72.00	
IV	2	73.10		77.64		75.73		79.98	
Microsatellite instability									
MSS	16	67.31	0.431	67.58	0.141	63.52	0.093	67.32	0.189
MSI	6	72.15		73.71		72.36		74.37	

[a] ANOVA test

Analysis of LINE-1 methylation and clinicopathological features using FFPE tissues

It was previously reported that LINE-1 hypomethylation is associated with tumor metastasis because it promotes liver metastasis in colorectal cancer [50]. One of results from our analysis using frozen tissues was that LINE-1 hypomethylation was associated with the presence of lymphatic invasion. In gastric cancer, there are several patients who are diagnosed with early gastric cancer with high number of lymph node metastasis, and there are also patients with advanced gastric cancer who have no lymph node metastasis. Therefore, we used 25 FFPE tumor tissues including 13 tissues from advanced gastric cancer without lymph node metastasis (T3/4N0) and 12 tissues from early gastric cancer with > 7 regional lymph node metastases (T1N3). When we compared LINE-1 methylation level between two groups, there was no significant difference ($p = 0.211$, Figure 28). Accordingly, to assess if LINE-1 methylation is related to certain other clinicopathological features, we further analyzed correlation between the methylation level and other clinicopathological parameters. As shown in Figure 29, LINE-1 methylation was significantly different according to tumor location ($p = 0.008$) and venous invasion ($p = 0.017$). In analysis of LINE-1 methylation level at each CpG site (Table 7), the methylation level at CpG1 was significantly lower in patients older than 57 years than in patients younger than 57 years and also significantly different according to the tumor location. In particular, the methylation levels at CpG1 and CpG2 were significantly lower in the lower third region than in middle third region. In case of venous invasion, the significant differences were shown at CpG2, CpG3 and CpG4.

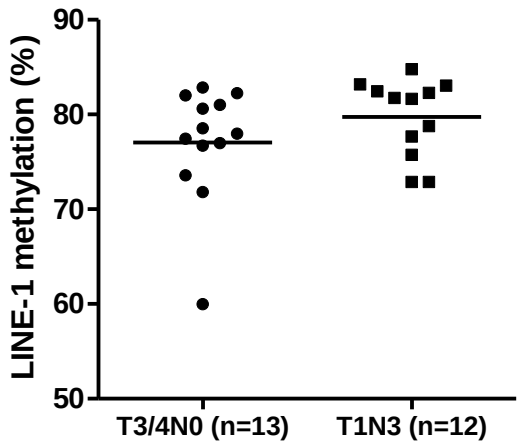


Figure 28. LINE-1 methylation levels between T3/4N0 and T1N3 tissues

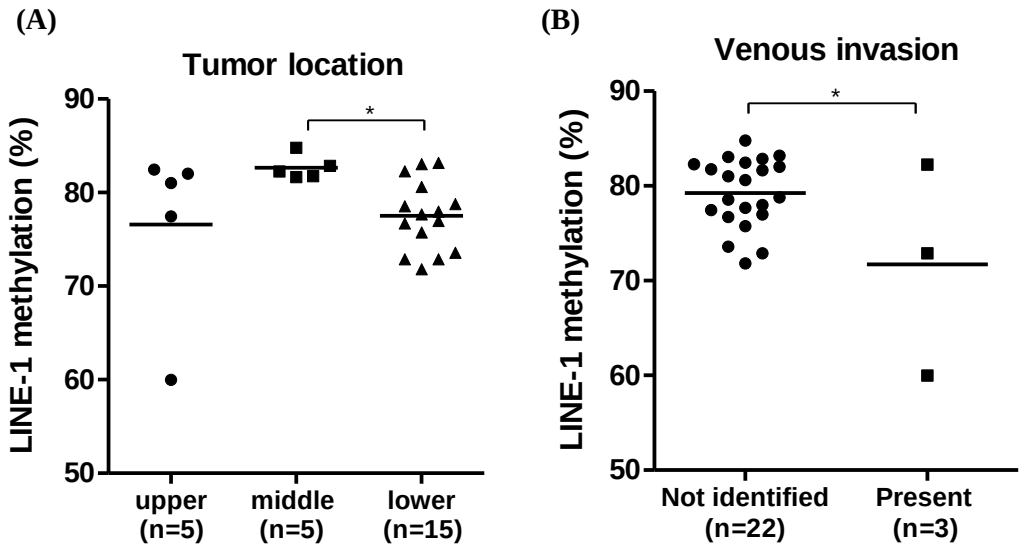


Figure 29. LINE-1 methylation levels according to tumor location (A) and venous invasion (B)

Table 7. The methylation level of LINE-1 at four CpG site according to clinicopathological characteristics of FFPE tumor tissues

Characteristic	No	LINE-1 methylation level (%)							
		CpG1	*P* value	CpG2	*P* value	CpG3	*P* value	CpG4	*P* value
Type									
T3/4N0	13	73.78	0.207	73.64	0.250	76.84	0.446	83.98	0.226
T1N3	12	77.26		75.57		78.91		87.31	
Age									
≤57	14	77.85	0.043	74.57	0.997	79.09	0.293	85.56	0.991
>57	11	72.40		74.56		76.23		85.60	
Gender									
Male	12	75.25	0.889	74.23	0.707	76.88	0.500	85.16	0.774
Female	13	75.64		74.87		78.72		85.96	
WHO classification									
Differentiated	6	71.51	0.103	74.04	0.870	75.71	0.384	87.09	0.487
Undifferentiated	15	76.97		74.40		78.70		84.60	
Lauren classification									
Intestinal	8	71.58	0.090	74.28	0.955	76.07	0.518	87.35	0.300
Diffuse	15	76.55		74.18		77.96		84.14	
Tumor location									
Upper	5	75.44	0.014[a]	73.59	0.156[a]	75.17	0.252[a]	82.11	0.367[a]
Middle	5	82.75	0.002[b]	77.73	0.008[b]	81.95	0.080[b]	88.19	0.319[b]
Lower	15	73.02		73.83		77.35		85.87	
Lymphatic invasion									
Not identified	11	75.68	0.883	74.01	0.563	78.12	0.856	85.54	0.982
Present	14	75.27		75.00		77.62		85.61	

Venous invasion									
Not identified	22	76.11	0.189	75.17	0.042	79.10	0.007	86.67	0.025
Present	3	70.56		70.10		68.56		77.58	
Perineural invasion									
Not identified	12	77.65	0.120	75.24	0.440	78.52	0.631	85.69	0.939
Present	13	73.41		73.94		77.21		85.47	
TNM stage									
II	23	75.49	0.919	74.82	0.309	77.99	0.703	86.02	0.274
IV	2	74.97		71.67		76.07		80.47	
Microsatellite instability									
MSS	14	75.15	0.379	73.66	0.826	77.21	0.852	83.57	0.555
MSI-high	3	71.06		74.33		76.34		86.53	

[a] ANOVA test

[b] Unpaired t-test (middle vs. lower)

LINE-1 methylation and venous invasion of gastric cancer

In both frozen and FFPE tumor tissues, LINE-1 was significantly hypomethylated in the presence of venous invasion compared to those in the absence of venous invasion. To evaluate if LINE-1 hypomethylation can be an epigenetic marker for venous invasion of gastric cancer, we additionally analyzed its methylation status in combined frozen and FFPE tumor tissues. We also combined the methylation levels at CpG1 and CpG4, since it was reported that the combination of LINE-1 methylation levels at CpG1 and CpG4 is useful marker for gastric cancer prognosis [54]. In the results, LINE-1 was significantly hypomethylated in the presence of venous invasion (Figure 30) compared to the absence of venous invasion in both analyses.

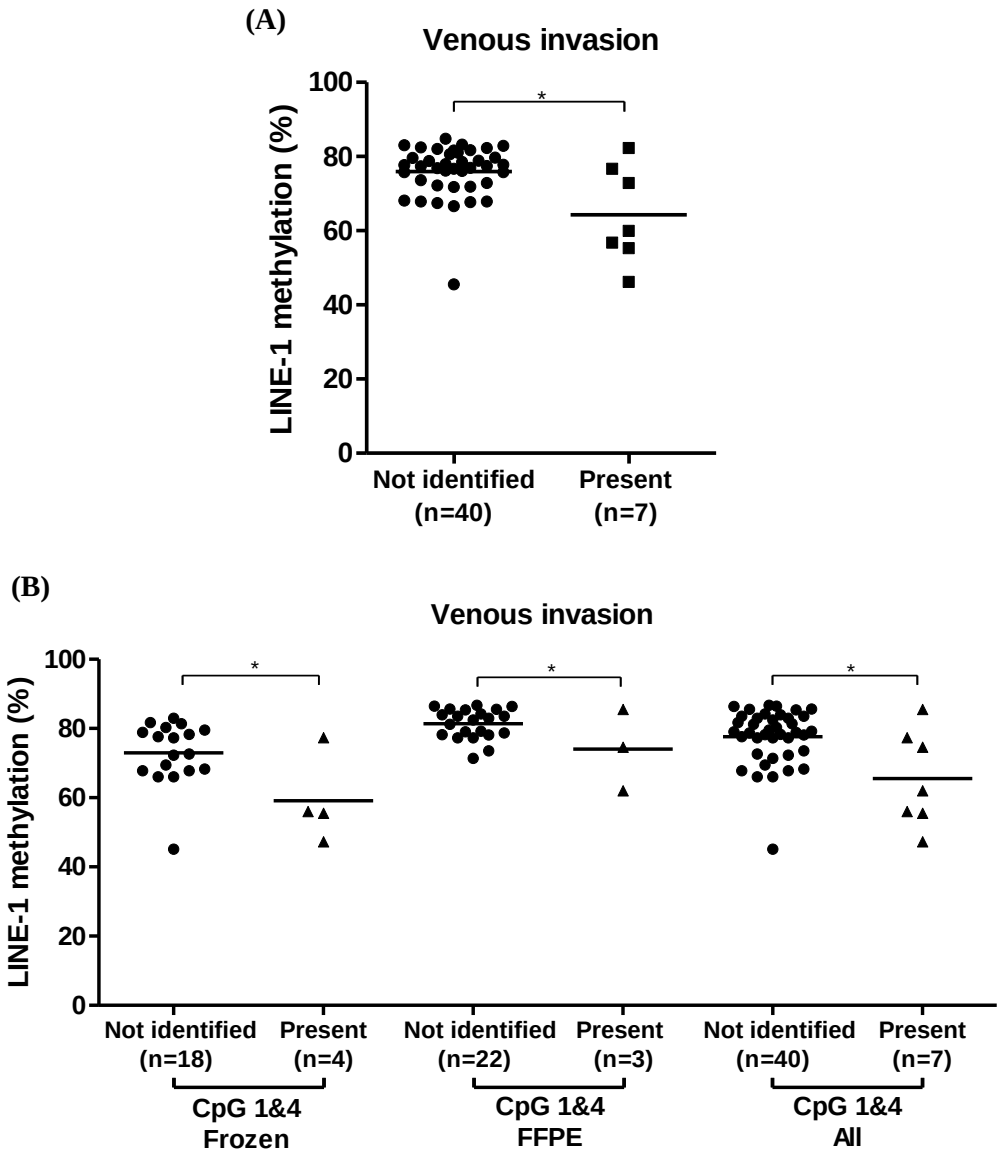


Figure 30. Combination analysis in venous invasion. Combination of the methylation levels in frozen and FFPE tissues (p = 0.001) (A) and combination of the methylation levels at CpG1 and CpG4 (frozen tissues; p = 0.018, FFPE tissues; p = 0.038, combined; p = 0.002) (B)

Discussion

1. miR-30a/target gene expressions and LINE-1 methylation in gastric cancer

In the present study, we used RNA sequencing data from TCGA, that large sample numbers of stomach adenocarcinoma are included, to confirm expression pattern of miR-30 family in gastric cancer. Among them, miR-30a was the most significantly downregulated compared with other members. Generally, hsa-miR-30a have two mature forms, miR-30a (miR-30a-5p) and miR-30a* (miR-30a-3p). In our *in vivo* experiments, we used stable cells overexpressing stem-loop sequence of miR-30a. It means two mature forms can affect the tumorigenesis in nude mice. When we validated their expressions in xenograft tumors using qRT-PCR, both forms were highly expressed in stable cells overexpressing miR-30a (data not shown). Other *in vitro* experiments were specifically performed about miR-30a-5p. Thus, we consider that miR-30a-5p has tumor suppressive functions in gastric cancer.

We identified novel candidate target genes of miR-30a, ITGA2, FBXO45, ADAM19 and SEC23A, by microarray technique. This study has focused on ITGA2 as a promising target of miR-30a because it was commonly upregulated in tumor tissues in both TCGA and our cohort. ITGA2 is one of family members of integrin, which is a transmembrane receptor for cell adhesion by forming a heterodimer with integrin beta subunits. ITGA2 expression can be epigenetically regulated by methylation or miRNAs, then it induces cell migration in cancer [56-59]. Shigeru et al. reported integrin α2 promotes migratory and invasive abilities of cancer cells by forming heterodimer with integrin β1 [60]. Another study showed that expression of α2β1 integrin is increased in peritoneal gastric cancer [61]. Recently, ITGA2 was revealed as an increased gene in advanced and metastatic gastric cancer as well [62].

In our previous study, ITGB1 was directly targeted by miR-29c and suppressed gastric carcinogenesis [63]. Accordingly, combination of mR-29c and miR-30a might be a crucial factor for gastric cancer treatment.

Although we have performed functional studies only for ITGA2, other three genes, FBXO45, ADAM19 and SEC23A may be promising targets for further study because they were also upregulated in gastric cancer compared to normal in TCGA data not in our tissue cohort. So far, few studies have revealed about their functions in cancer. Decreased FBXO45 expression is oncogenic in gastric cancer [64], but other two genes have been not reported about their tumor-associated functions in gastric cancer. Oncogenic ADAM19 is associated with metastasis in angiosarcoma [65], and SEC23A promotes secretome and metastasis of breast cancer cell [66].

Tissue samples from patients are obtained for various purposes, so they were preserved in various forms with/without pre-treatments according to the purposes. To pathologically examine gastric cancer tissues, formalin-fixation and paraffin-embedding are performed. After paraffin block preparation, sections are used for histological review. For gene- or protein-based analyses such as expression analysis, and pyrosequencing, tissue samples were usually stored in liquid nitrogen without any pre-treatment as soon as possible after gastrectomy.

In DNA methylation analyses, formalin fixation can affect bisulfite modification because formaldehyde can cause sequence artifacts such as cytosine deamination [67]. Thus, DNA methylation can be different according to tissue-storage conditions. In previous studies, certain clustered methylation patterns were differentially shown between frozen and FFPE tissues of colon [68], and the MGMT promoter was differentially methylated between two types of glioblastoma tissue samples [69]. In

gastric cancer, LINE-1 was significantly hypomethylated in fresh/frozen tissues than in paired FFPE tissues [54].

As a previous study, quantitative bisulfite pyrosequencing is one of useful methods to measure DNA methylation levels in biomarker studies [70]. For LINE-1 methylation analysis, diverse methods, such as combined bisulfite restriction analysis, MethyLight and bisulfite pyrosequencing, have been used. Particularly, pyrosequencing is also the most useful to detect LINE-1 methylation accurately and reliably [71-73].

2. Evaluation of miR-30a and LINE-1 as markers for clinicopathological features of gastric cancer

H. pylori causes precancerous progression and carcinogenesis of gastric cancer, and especially in 60% of gastric adenocarcinoma, intestinal type gastric cancer is triggered through intestinal metaplasia and dysplasia after *H. pylori* infection [74]. It also induces MSI of gastric cancer [75], and MSI with *H. pylori* infection may induce gastric carcinogenesis [76]. miR-30a downregulation causes HNF4γ upregulation in intestinal metaplasia [33]. In our results, expression of miR-30a and ITGA2 is considered as genes which may be affected by *H. pylori* infection, MSI and histological type of gastric cancer. We found that miR-30a was downregulated in *H. pylori* infected, MSI-high and intestinal type gastric cancer. miR-30a was recently reported as a tumor suppressor in *H. pylori*-induced gastric cancer [77]. In contrast, ITGA2 was upregulated in MSI-high and intestinal type gastric cancer. Therefore, miR-30a-ITGA2 dysregulation may contribute to MSI-high gastric cancer affected *H. pylori* after miR-30a-HNF4γ-affected metaplasia, especially intestinal type of gastric cancer, because both ITGA2 mRNA and cytoplasmic ITGA2 were highly identified in intestinal type.

LINE-1 methylation status can be a marker for detecting early gastric cancer as well as advanced gastric cancer using FFPE tissues [54, 78]. Particularly, it has been identified that LINE-1 methylation can be a marker for venous invasion in frozen tissues of lung cancer [79] and in serum samples of hepatocellular carcinoma [80]. Vascular invasion can induce tumor angiogenesis, lymph node metastasis and distant metastasis in gastric cancer [81]. Thus, finding biomarkers for vascular invasion in gastric cancer are needed to predict tumor metastasis. In our result, LINE-1

hypomethylation can be a marker for venous invasion in irrespective of tissue types. Further studies may be needed to assess the utility of serum samples for non-invasive detection of venous invasion.

3. Conclusion

To the best our knowledge, the present study is the first study which identified novel candidate target genes of miR-30a including ITGA2 based on microarray technique and which evaluated the association between miR-30a-ITGA2 expression and various clinical factors in gastric cancer. In conclusion, each miR-30a and ITGA2 has a role of regulating gastric cancer cell growth and motility. Clinicopathologically, miR-30a-ITGA axis can be an independent biomarker for *H. pylori* infection and MSI of gastric cancer, and both ITGA2 mRNA and cytoplasmic ITGA2 are upregulated in intestinal type gastric cancer. This study highlighted that miR-30a-ITGA2 may be a therapeutic target and biomarker for prediction of gastric carcinogenesis and prognosis.

Our LINE-1 study is also the first to investigate associations between LINE-1 methylation status and clinicopathological factors using both frozen and FFPE tissues of gastric cancer. Our limitations are as follows: (i) FFPE tissues consist of specific T and N stages such as T1N3 and T3/4N0. (ii) Small sample sizes were used. Therefore, validation study using more samples is needed. In conclusion, this study validated the methylation pattern of LINE-1 in frozen tissue samples which were not paired with FFPE tissue samples, and LINE-1 methylation status was related to several clinicopathological parameters in each sample set. The LINE-1 methylations in frozen tissues were different according to gender, differentiation, lymphatic and venous invasions, and the LINE-1 methylations in FFPE tissues were different according to tumor location and venous invasion. This study implies that LINE-1 methylation can be a marker for several clinicopathological features in each tissue type and for venous invasion in both gastric cancer tissue types.

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요약(국문초록)

배경: 위암에서 마이크로RNA (miRNA) 나 DNA 메틸화와 같은 후성적 유전자 변화는 암세포의 특성을 조절하는 주요 인자이며, 여러 유전자의 발현을 조절함으로써 세포의 증식, 분화 및 세포사멸 등을 조절할 수 있다. miRNA와 같은 small RNA는 Long interspersed nuclear element-1 (LINE-1) 이라는 전이성 유전인자의 역전위에 의해 발생되기도 하는데, DNA 메틸화에 의해 LINE-1 과 같은 전이성 유전인자가 억제될 수 있다. miR-30a는 위암에서 저발현 되어 있지만 아직 그 생물학적 기능과 임상적인 의의가 많이 밝혀져 있지 않고, LINE-1은 주로 암에서 저메틸화 되어 있다고 알려져 있는데 특히 LINE-1 억제가 miR-30a의 발현 증가와 관련이 있다는 보고가 있다.

목적: 본 연구는 위암에서 miR-30a 발현과 LINE-1 메틸화 패턴을 확인하고 miR-30a의 위암 특이적인 타겟 유전자를 찾음으로써 그 생물학적 기능을 조사하고자 하였다. 또한 위암 환자에서의 임상적 의의를 평가함으로써 miR-30a와 LINE-1의 진단 또는 예후 마커로서의 이용가능성을 평

가하고자 하였다.

방법: miR-30a와 타겟 유전자의 발현 확인을 위해 위암 환자 조직을 이용한 qRT-PCR을 시행하였고, TCGA와 NCBI GEO 데이터를 통해 발현을 검증하였다. 위암세포주에 miR-30a-5p를 과발현 또는 억제하여 생물학적 기능 연구를 시행하였으며, *in vivo* 실험을 위해 stable cell line을 제작하였다. Microarray 기법을 통해 miR-30a의 위암 특이적 타겟 유전자를 확인 후 siRNA를 이용하여 *in vitro* 실험을 시행하였다. LINE-1 메틸화 연구에서는 파라핀 조직 및 동결 조직을 이용하여 pyrosequencing을 통해 4개의 LINE-1 CpG 부위의 메틸화 정도를 분석하였다. 최종적으로 유전자 발현 및 메틸화 정도에 따른 위암 환자의 임상병리데이터를 분석하였다.

결과: 위암 조직에서 miR-30a가 저발현 되어 있었고, LINE-1은 저메틸화 되어 있었다. miR-30a가 ITGA2 유전자의 3′ UTR 부위에 결합하여 직접적으로 발현을 조절함을 확인하였고, 위암 세포주에 miR-30a-5p를 과발현 시키거나 ITGA2를 억제했을 때 세포 성장과 이동성, 콜로니 형성 능력이 모두 감소하였다. 특히 miR-30a와 ITGA2가 *Helicobacter pylori* 감염이 있는 위암 환자 및 Microsatellite instability가 높은 위암 환자, 그리고 장

형 위암 (Intesinal type) 에서 통계적으로 유의한 관련성을 보였다. 동결 조직과 파라핀 조직에서 LINE-1 메틸화를 분석했을 때, 두 종류의 조직에서 LINE-1 메틸화가 다르게 나타났다. 동결 조직에서는 LINE-1 메틸화가 환자의 성별, 조직의 분화도, 종양의 림프 및 정맥 침윤과 관련이 있었고, 파라핀 조직에서는 종양 위치 및 정맥 침윤과 관련이 있었다.

결론: 본 연구 결과 위암에서 miR-30a 발현이 감소되어 있고 miR-30a와 그 타겟 유전자인 ITGA2가 위암 세포의 특성을 조절하였다. miR-30a를 조절하는 인자 중 하나인 LINE-1 또한 분석한 조직 종류에 따라 몇 가지 임상병리학적 특성에 따른 메틸화 마커로서 사용될 수 있고, 조직 종류에 관계 없이 종양의 정맥 침윤 마커로서 사용될 수 있다. 그러므로 miR-30a-ITGA2 및 LINE-1이 위암의 치료 및 예후 예측을 위한 전략이 될 수 있을 것이다.

주요어: 후성적 조절, miR-30a, 종양 억제 유전자, ITGA2, LINE-1, 정맥 침윤, 위암

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